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Patents ADP number *(if you know it)*

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METHODS

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Description 84

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METHODS

This invention relates to a diagnostic method for the detection of a cytochrome *b* mutation in fungi that leads to resistance to strobilurin analogues or compounds in the same cross resistance group using any (or a) single nucleotide polymorphism detection technique, preferably using the amplification refractory mutation system (ARMS). The invention also relates to mutation specific primers for use in the method and to diagnostic kits containing these primers. The invention further relates to the identification of a specific mutation in the fungal cytochrome *b* gene which results in the resistance of fungi containing the said mutation to strobilurin analogues or compounds in the same cross resistance group.

Description

The widespread use of fungicides in agriculture is a relatively recent phenomenon, and most of the major developments have taken place during the last 40 years. Previously, farmers often ignored or did not recognise the effect that fungal pathogens had on the yield and quality of their crops. Nowadays, however, these losses are unacceptable, and farmers rely on the use of fungicidal chemicals to control fungal diseases. As a consequence, commercial fungicides have become an important component of the total agrochemical business, with world-wide sales in 1996 of about $5.9 billion, equivalent to 18.9% of the total agrochemical market (Wood Mackenzie, 1997a 'Agchem products- The key agrochemical product groups', in Agrochemical Service, Update of the Products Section, May 1997, 1-74). A large number of fungicides are already available to the farmer; a recent edition of The Pesticide Manual (Tomlin, 1994 10th Edition, British Crop Protection Council, Farnham, UK, and the Royal Society of Chemistry, Cambridge, UK) contains 158 different fungicidal active ingredients in current use. Nevertheless, further industrial research aimed at the discovery and development of new compounds is extremely intensive and product management procedures are extremely important in securing the best and

resistance management strategies when fungicides with new modes of action are

introduced (Fungicide Resistance Management : Into The Next Millenium (Russell) 1999, in Pesticide Outlook, October 1999 (213-215).

The strobilurin analogues constitute a major new series of agricultural fungicides which are considered the most exciting development on the agricultural fungicide scene since the discovery of the 1,2,4-triazoles in the 1970s.

The fungicidal activity of the strobilurin analogues is a result of their ability to inhibit mitochondrial respiration in fungi. More specifically, it has been established that these compounds have a novel single site mode of action, exerting their effect on fungi by blocking the ubiquinol:cytochrome c oxidoreductase complex (cytochrome *bc*1) thus reducing the generation of energy rich ATP in the fungal cell (Becker et al FEBS Letts. 132 329-33). This family of inhibitors prevents electron transfer at the ubiquinone redox site Q_o on the dimeric cytochrome *b* protein (Esposti et al 1993 Biochim. et Biophys Acta 243-271). Unlike many mitochondrial proteins, the cytochrome *b* protein is mitochondrially encoded.

Reports in the literature show that specific amino acid changes at the cytochrome *b* target site can affect the activity of strobilurin analogues. In depth mutagenesis studies in *Saccharomyces cerevisiae* (JP Rago et al 1989 J. Biol. Chem. 264, 14543-14548), mouse (Howell et al 1988 J. Mol. Biol. 203, 607-618), *Chlamydomonas reinhardtii* (Bennoun et al 1991 Genetics 127, 335-343) and *Rhodobacter spp* (Daldal et al 1989 EMBO J. 3951-3961) have been carried out. Relevant information was also gathered from studying the natural basis for resistance to strobilurin analogues in the sea urchin *Paracentrotus lividus* (Esposti et al 1990 FEBS 263, 245-247) and the Basidiomycete fungi *Mycena galopoda* and *Strobilurus tenacellus* (Kraiczy et al 1996 Eur. J. Biochem. 235, 54-63), both of which produce natural variants of the strobilurin analogues. There are two distinct regions of the cytochrome *b* gene where amino acid changes have a dramatic effect on strobilurin analogue activity. These areas cover amino acid residues 125-148 and 250-295 (based on *S.cerevisiae* residue numbering system). More precisely amino acid changes at residues 126, 129, 132, 133, 137, 142, 143, 147, 148, 256, 275 and 295 have been shown to give rise to resistance to strobilurin analogues (Brasseur et al 1996 Biochim. Biophys. Acta 1275, 61-69 and Esposti et al (1993) Biochimica et Biophysica Acta, 1143, 243-271).

The present invention identifies for the first time the key importance of one of these mutations in cytochrome *b* gene of field isolates of important plant pathogenic fungi showing resistance to a strobilurin analogue or a compound in the same cross resistance group.

According to a first aspect of the invention we provide a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

In the present invention we have now devised novel diagnostic methods for the detection of a point mutation in a fungal cytochrome *b* gene based on single nucleotide polymorphism detection methods including allele specific amplification. It will be apparent to the person skilled in the art that there are a large number of analytical procedures which may be used to detect the presence or absence of variant nucleotides at one or more polymorphic positions according to the invention. In general, the detection of allelic variation requires a mutation discrimination technique, optionally an amplification reaction and optionally a signal generation system. Many current methods for the detection of allelic variation are reviewed by Nollau et al, Clin. Chem. 43, 1114-1120, 1997 and in standard textbooks, for example 'Laboratory Protocols for Mutation Detection', Ed, bu U. Landegren, Oxford University Press, 1996 and 'PCR' 2nd Edition by Mewton and Graham, BIOS Scientific Publishers limited, 1997. Allele specific amplification reactions include primer based methods including PCR based methods and more specifically, allele specific polymerase chain reaction (PCR) extension (ASPCR) and specifically ARMS (Amplification Refractory Mutagenesis System) wherein the mutation gives rise to resistance to a strobilurin analogue and these are particularly preferred for use in the methods of the present invention. The methods of the invention also include indiscriminate PCR reaction

strobilurin analogues or any other compound in the same cross resistance group.

Robust tests have been developed for the detection of this mutation in

fungal plant pathogens. Compounds may be considered to be in the same cross resistance group when the resistance mechanism to one compound also confers resistance to another, even when the modes of action are not the same. The technique of ASPCR is described in US Patent No. 5639611 and the ARMS technique is described fully in European Patent No. EP 332435.

Other single polymorphism detection techniques which may be used to detect mutations include, for example, restriction fragment length polymorphism (RFLP), single strand conformation polymorphism, multiple clonal analysis, allele-specific oligonucleotide hybridisation, single nucleotide primer extension (Juvonen et al, (1994) Hum Genet 93 16-20; Huoponen et al, (1994) Hum Mutat 3 29-36; Mashima et al (1995), Invest Opthelmol. Vision. Sci 36,1714-20; Howell et al (1994) Am J Hum Genet. 55 203-206; Koyabashi et al; (1994) Am. J. Hum. Genet. 55 206-209; Johns and Neufeld (1993) Am J Hum Genet 53 916-920; Chomyn et al, (1992) Proc. Natl. Acad. Sci USA 89 4221-4225) and Invader ™ technology (available from Third Wave Technologies Inc. 502 South Rosa Road,Madison, WI 53719 USA).

The use of PCR based detection systems is preferred.

According to a preferred embodiment of the first aspect of the invention we provide a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of said mutation in said nucleic acid.

The detection of the amplicon generated during the PCR reaction may be directly dependent on the extension of a primer specific for the presence of the mutation i.e. where primer extension is dependent on the presence of the mutation and hence an amplicon is generated only when the primer binds and/or is extended when the mutation is present (as is the case with ARMS technology), similarly it may be directly dependent on the extension of a primer specific for the absence of the mutation e.g. wild type sequence or may be directly linked to the PCR extension

product containing the mutant DNA sequence i.e. where the detection is of an amplicon comprising the mutant DNA sequence. The first alternative is particularly preferred.

The amplicon can be from any PCR cycle and this includes a first allele specific primer extension product.

In a further preferred embodiment the invention provides a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with an appropriate diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended either when the said mutation is present in the sample or when wild type sequence is present; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

In a further preferred embodiment the invention provides a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

According to a particularly preferred embodiment of the first aspect of the invention we provide a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises

agent for polymerisation, such that the diagnostic primer is extended only when the

said mutation by reference to the presence or absence of a diagnostic primer extension product.

As used herein the term diagnostic primer is used to indicate the primer which is used specifically to identify the presence or absence of a mutation or wild type sequence and the term common primer is used to denote a primer binding to the opposite strand of DNA to the diagnostic primer and 3' to the region recognised by that diagnostic primer and which, by acting with said diagnostic primer allows amplification of the intervening tract of DNA during the PCR. Where the diagnostic primer is an ARMS primer it can have a 3' mismatch when compared to the mutant or wild type sequence.

In this and all further aspects and embodiments of the invention it is preferred that the extension of the primer extension product is detected using a detection system which is an integral part of either the diagnostic primer or the common primer on the opposite strand. This is described more fully herein.

The methods of the invention are particularly suitable for the detection of mutations in a mitochondrial gene which encodes a protein which is a target for a fungicide, more especially for the detection of mutations in a fungal cytochrome *b* gene where said mutations result in the inhibition of fungicide activity but still allow the respiration process to occur and most preferably wherein said mutation in the fungal cytochrome *b* gene results in one of the following amino acid substitutions: $A_{126}T$, $F_{129}L$, $Y_{132}C$, $C_{133}Y$, $G_{137}R/S/E/V$, $W_{142}T/K$, $G_{143}A$, $I_{147}F$, $T_{148}M$, $N_{256}Y/K/I$, $L_{275}F/S/T$ or $L_{295}F$, the presence of which give rise to fungal resistance to strobilurin analogues or any other compound in the same cross resistance group wherein the residue identification is based on the *S. cerevisiae* residue numbering system for cytochrome *b*.

The strobilurin analogues and compounds in the same cross resistance group include for example, azoxystrobin, picoxystrobin, kresoxim-methyl, trifloxystrobin, famoxadone and fenamidone.

We have found that the position in the fungal cytochrome *b* nucleic acid corresponding to the 143rd codon/amino acid in the cytochrome *b* of *Saccharomyces cerevisiae* sequence is a key determinant of fungal resistance to strobilurin analogues or any other compound in the same cross resistance group in field isolates of

strobilurin analogue resistant plant pathogenic fungi. The methods of the invention described herein are particularly suitable for the detection of a mutation at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 where the glycine residue is replaced by another amino acid which inhibits the activity of strobilurin analogues or any other compound in the same cross resistance group and results in a resistant phenotype in the fungi carrying the mutant cytochrome *b* gene thereby giving rise to fungal resistance to strobilurin analogues or any other compound in the same cross resistance group.

The method is preferably used for the detection of a mutation resulting in the replacement of said glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 with an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan and most preferably alanine.

In a yet further preferred embodiment of the first aspect of the invention we now provide a method for the detection of a mutation in a fungal cytochrome *b* gene resulting in a glycine to alanine replacement at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 ($G_{143}A$) in the encoded protein thereby giving rise to fungal resistance to strobilurin analogues or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

The mutation in the fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein is usually a guanine to cytosine base change at the second position (base) of the codon and the detection of this single nucleotide polymorphism is preferred for all aspects and embodiments of the invention described herein.

In a still further preferred embodiment of the first aspect of the invention we now provide a diagnostic method for the detection of a mutation in a fungal

[illegible] reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer in the presence of appropriate nucleoti[illegible]

triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of said mutation in said nucleic acid.

In a particularly preferred embodiment of the first aspect of the invention we now provide a diagnostic method for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the mutation resulting in a $G_{143}A$ replacement in the encoded protein in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when a mutation is present in the sample resulting in a $G_{143}A$ replacement in the encoded protein; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

In a further particularly preferred embodiment of the first aspect of the invention we now provide a diagnostic method for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the mutation resulting in a $G_{143}A$ replacement in the encoded protein in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when a mutation resulting in a $G_{143}A$ replacement in the encoded protein is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

As used herein the term $G_{143}A$ is used to denote the substitution of a glycine residue by an alanine residue in a fungal cytochrome *b* sequence at the equivalent of the position of the 143rd codon/amino acid of the *Saccharomyces cerevisiae* cytochrome *b* sequence. This nomenclature is used for all the other residue changes quoted herein i.e. all positions are quoted relative to the *S. cerevisiae* cytochrome *b* protein sequence. The *S. cerevisiae* cytochrome *b* gene and protein sequences are available on the EMBL sequence database (See EMBL ACCESSION NO. X84042). The skilled man will appreciate that the precise length and register of equivalent proteins from different species may vary as a result of amino or carboxy terminal and/or one or more internal deletions or insertions. Since the amino acid tract

containing the residue corresponding to G_{143} in *S.cerevisiae* is well conserved (Widger *et al.* Proc.Nat.Acad.Sci., U.S.A. **81** (1984) 674-678) it is straightforward to identify the precisely corresponding residue in a newly obtained fungal cytochrome b sequence either by visual inspection or use of one of several sequence alignment programmes including Megalign or Macaw. Though designated G_{143} in this application, because of positional and functional equivalence, the precise position of this glycine in the new cytochrome *b* may not be the 143rd residue from its amino terminal end. In all aspects of the invention described herein the positions in the cytochrome *b* sequence are preferably as defined relative to the *Saccharomyces cerevisiae* cytochrome *b* sequence provided in EMBL ACCESSION NO. X84042

According to one aspect of the invention there is provided a method for the diagnosis of a single nucleotide polymorphism in a fungal cytochrome *b* gene which method comprises determining the sequence of the fungal nucleic acid at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein and determining the resistance status of the said fungi to a strobilurin analogue or a compound in the same cross resistance group by reference to a polymorphism in the cytochrome *b* gene.

The position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein is preferably as defined by the positions in EMBL ACCESSION NO. X84042.

In all aspects and embodiments of the invention described herein it is preferred that only one base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein shows a mutation i.e there is a single nucleotide polymorphism occurring at one position only and it is further preferred that it is at the first or second base of the triplet and most especially is at the second base in the triplet.

According to a preferred embodiment of this aspect of the invention there is

nucleic acid at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae*

cytochrome *b* residue 143 in the cytochrome *b* protein and determining the resistance status of the said fungi to a strobilurin analogue or a compound in the same cross resistance group by reference to a polymorphism in the cytochrome *b* gene.

The position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein is preferably as defined by the positions in EMBL ACCESSION NO. X84042.

In an embodiment of the above aspect of the invention the method for diagnosis described herein is one in which the single nucleotide polymorphism at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein is presence of G and/or C.

First position	Second position		Third position
5'end	C	G	3'end
G	Alanine	Glycine	U
	Alanine	Glycine	C
	Alanine	Glycine	A
	Alanine	Glycine	G

Table 1: codon usage

A glycine to alanine point mutation demands a G to a C change at the second base of the codon. Other mutations may also arise at the 3rd position in the codon due to degeneracy in genetic code for alanine and glycine (see Table 1) but this is readily taken into consideration when designing the diagnostic primer. The diagnostic primer is preferably an ARMS primer. (The concept of ARMS primers is described fully in Newton et al, Nucleic Acid Research 17 (7) 2503-2516 1989). As a result ARMS primers can be designed for the detection of the $G_{143}A$ point mutation given only sequence information on the wild type, strobilurin analogue sensitive, cytochrome *b* gene. There is no need to have access to a resistant isolate in new fungi of interest resulting from a $G_{143}A$ mutation. Some examples of relevant plant pathogenic fungi are listed in Table 2. This list is not meant to be in any way to be exclusive. The

skilled plant pathologist will be able to readily identify those fungi to which the methods of this invention are relevant.

	Examples of species in which $G_{143}A$ can be assayed :
1	*Plasmopara viticola*
2	*Erysiphe graminis f.sp. tritici/hordei*
3	*Rhynchosporium secalis*
4	*Pyrenophora teres*
5	*Mycosphaerella graminicola*
6	*Mycosphaerella fijiensis* var. *difformis*
7	*Sphaerotheca fuliginea*
8	*Uncinula necator*
9	*Colletotrichum graminicola*
10	*Pythium aphanidermatum*
11	*Colletotrichum gloeosporioides*
12	*Oidium lycopersicum*
13	*Leveillula taurica*
14	*Pseudoperonospora cubensis*
15	*Alternaria solani*
16	*Cercospora arachidola*
17	*Rhizoctonia solani*
18	*Venturia inaequalis*
19	*Magnaporthe grisea*
20	*Phytophthora infestans*
21	*Mycosphaerella musicola*

Table 2: Example of species where $G_{143}A$ can be assayed

The methods of the invention described herein are particularly useful in

secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis var. *difformis, Sphaerotheca fuliginea, Uncinula necator*

Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea ,Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.

In a further aspect the invention provides a method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

In a further preferred embodiment of this aspect the invention provides a method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

In a preferred embodiment of this aspect of the invention the presence or absence of a single nucleotide polymorphism at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein in the cytochrome *b* gene in fungal nucleic acid is identified using any (or a) single nucleotide polymorphism detection techniques .

The invention further provides a fungal DNA sequence encoding all or part of a wild type cytochrome *b* protein wherein said DNA sequence encodes a glycine

residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the wild type protein wherein said sequence is obtainable from a fungus selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctania solani, Mycosphaerella musicola and Cercospora arachidola.*

The fungal DNA sequences according to the above aspects of the invention preferably comprises around 30 nucleotides on either or both sides of the position in the DNA corresponding to one or more of the bases in the triplet, preferably corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein since this extent of nucleic acid provides the skilled man with all information necessary to design species and mutation specific reagents and/or methods for use in all single nucleotide polymorphism detection techniques. As used herein the term around 30 means that the sequence may comprise up to 30 nucleotides, for example 5, up to 10, 15, 20, or 25 nucleotides or may comprise more than 30 nucleotides.

As used herein in connection with all DNA and protein sequences the term 'all or part of' is used to denote a DNA sequence or protein sequence or a fragment thereof. A fragment of DNA or protein may for example be 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95%of the length of the whole sequence.

It will be evident to the man skilled in the art that both samples containing genomic (mitochondrial) and cDNA may be analysed according to the invention. Where the sample contains genomic DNA intron organisation needs to be taken into account when using the sequence information. Examples of wild type fungal DNA

form a further aspect of the invention.

Species	Sequence
Plasmopara viticola (cDNA & genomic)	5'TTTTGCCTTGGGGACAAATGAGTTTTTGGGGTGCAAC AGTTATTACAAATTTATTCTCGGC 3'
Erysiphe graminis f.sp. tritici/hordei cDNA & (cDNA & genomic)	5'TATTGCCATACGGGCAGATGAGCCACTGGGGTGCAAC CGTTATCACTAACCTAATGAGCGC 3'
Rhynchosporium secalis (cDNA & genomic)	5'TGCTTCCTTATGGACAGATGTCTTTATGAGGTGCCACA GTTATAACTAATCTTATGAGTGC 3'
Pyrenophora teres (cDNA)	5'TTTTACCCTACGGGCAAATGAGCCTTTGAGGTGCTAC AGTTATTACTAACCTTATGAGTGC 3'
Pyrenophora teres (genomic)	5'TTTTACCCTACGGGCAAATGAGCCTTTGAGGTGAAAT ATTTGCCTCAAATGTATAACTAAT 3'
Mycosphaerella graminicola (cDNA & genomic)	5'TATTACCTTATGGTCAAATGTCTTTATGAGGAGCAAC AGTTATAACTAACTTATTGAGTGC 3'
Mycosphaerella fijiensis var. difformis (cDNA & genomic)	5'TTTTACCTTATGGTCAAATGTCTTTATGAGGAGCTACA GTTATAACTAATTTAATGAGCGC 3'
Sphaerotheca fuliginea (cDNA)	5'TACTTCCCTTCGGTCAAATGTCGCTCTGGGGTGCAACC GTTATTACTAACCTTATGAGCGC 3'
Sphaerotheca fuliginea (genomic - *6bp upstream available)	5' *TCTGGGGTGCAACCGTTAAGTAATAGCGGTTGTAAAA
Uncinula necator (cDNA)	5'TTTTACCCTACGGGCAGATGAGCCTATGGGGTGCAAC CGTTATTACTAACCTTATGAGCGC 3'
Uncinula necator (genomic - *10bp upstream available)	5'*AGCCTATGGGGTGCAACCGTTAAGTAGGTAATAGCG GTTGA 3'
Colletotrichum graminicola (genomic & cDNA)	5'TTTTACCTTACGGACAAATGTCATTATGAGGTGCTAC AGTTATTACTAACCTTATAAGTGC 3'
Pythium aphanidermatum (genomic & cDNA)	5'TATTACCTTGGGGTCAAATGAGTTTTTGGGGTGCTACT GTTATTACTAATTTATTTTCAGC 3'
Colletotrichum gloeosporioides (genomic & cDNA)	5'TTTTACCTTATGGACAAATGTCATTATGAGGTGCAAC AGTTATTACTAACCTTATAAGTGC 3'
Oidium lycopersicum (cDNA)	5'TTTTACCCTACGGGCAGATGAGCCTGTGGGGTGCAAC CGTTATTACTAACCTTATGAGCGC 3'
Leveillula taurica (cDNA)	5'TTTTACCATACGGACAAATGTCATTATGAGGTGCAAC AGTTATTACTAACCTTATGAGTGC 3'
Pseudoperonospora cubensis	5'TTTTACCTTGGGGACAAATGAGTTTTTGGGGTGCAAC

(cDNA & genomic)	TGTTATTACTAATTTATTTTCTGC 3'
Alternaria solani (cDNA & genomic)	5'TTCTTCCTTATGGGCAAATGTCTTTATGAG**G**TGCTACA GTTATTACTAACCTTATGAGTGC 3'
Cercospora arachidola (cDNA & genomic)	5'TATTACCTTATGGACAAATGTCATTATGAG**G**AGCTAC AGTTATTACTAATTTATTATCTGC 3'
Rhizoctonia solani (cDNA)	5'TGCTTCCATACGGGCAAATGTCTCTGTGGG**G**TGCTAC AGTAATTACTAATTTACTTTCTGC 3'
Mycosphaerella musicola (genomic & cDNA)	5'TTTTACCTTATGGTCAAATGTCTTTATGAG**G**AGCTACA GTTATAACTAATTTAATGAGTGC 3'

Table 3

In the above table the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid wherein said replacement confers resistance to strobilurin analogues or a compound within the same cross resistance group is in bold and underlined.

The invention also extends to a fungal DNA sequence showing homology or sequence identity to said DNA sequences in Table 3 and covers for example, variations in DNA sequences found in different samples or isolates of the same species. These variations may, for example, be due to the use of alternative codon usage, varying intron/exon mitochondrial organisation and amino acid replacement.

In a further aspect the invention provides a fungal DNA sequence encoding all or part of a cytochrome *b* protein which, when said sequence is lined up against the corresponding wild type DNA sequence encoding a cytochrome *b* protein, is seen to contain a single nucleotide polymorphism mutation at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena*

fungal DNA sequence encoding all or part of a cytochrome *b* protein which, when said sequence is lined up against the corresponding wild type DNA sequence

encoding a cytochrome *b* protein, is seen to contain a single nucleotide polymorphism mutation at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

The fungal DNA sequence according to the above aspect of the invention preferably comprises around 30 nucleotides on either or both sides of the position in the DNA corresponding to one or more of the bases in the triplet, preferably corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein since this extent of nucleic acid provides the skilled man with all information necessary to design species and mutation specific reagents and/or methods for use in all single nucleotide polymorphism techniques. As used herein the term around 30 means that the sequence may comprise up to 30 nucleotides, for example 5, up to 10, 15, 20, or 25 nucleotides or may comprise more than 30 nucleotides.

The invention further provides a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein the presence of a mutation in said DNA confers resistance to a strobilurin analogue or a compound within the same cross resistance group, said mutation occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

In a preferred embodiment of this aspect the invention further provides a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein the presence of a mutation in said DNA confers resistance to a strobilurin analogue or a compound within the same cross resistance group, said mutation occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

In the above aspects of the invention the mutation occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein is preferably a guanine to cytosine base change.

The fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein the presence of a mutation in said DNA confers resistance to a strobilurin analogue or a compound within the same cross resistance group, according to the above aspects of the invention is preferable obtainable form a fungus selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea ,Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

The invention extends also to DNA sequences comprising all or part of the sequences provided in Table 3 wherein the residue at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein is a cytosine residue.

The invention also extends to a fungal DNA sequence showing homology or sequence identity to said DNA sequence containing said polymorphism and covers for example, variations in DNA sequences found in different samples of the same species. These variations may, for example, be due to the use of alternative codon usage, varying intron/exon mitochondrial organisation and amino acid replacement.

The invention further provides a computer readable medium having stored thereon any of the sequences described and claimed herein and including all or part of

strobilurin analogue or any compound in the same cross resistance group; all or part of a DNA or protein sequence encoding a mutant cytochrome *b* protein wherein said

protein confers fungal resistance to a strobilurin analogue or a compound in the same cross resistance group from a fungus selcted from the group *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea ,Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola,* with the proviso that said DNA or protein sequence is not the Mycena galopoda cytochrome b sequence; all or part of a DNA or protein sequence encoding a wild type cytochrome *b* sequence from a fungus selected from the group *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, , Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, , Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola;* or any allele specific oligonucleotide; allele specific oligonucleotide probe, allele specific primer, common or diagnostic primer disclosed herein.

The computer readable medium may be used, for example, in homology searching, mapping, haplotyping, genotyping or any other bioinformatic analysis. Any computer readable medium may be used, for example, compact disk, tape, floppy disk, hard drive or computer chips.

The polynucleotide sequences of the invention, or parts thereof, particularly those relating to and identifying the single nucleotide polymorhisms identified herein, especially the G to C change in fungal cytochrome *b* causing the $G_{143}A$ change in the encoded protein, represent a valuable information source. The use of this information source is most easily facilitated by storing the sequence information in a computer readable medium and then using the information in standard bioinformatics programs. The polynucleotide sequences of the invention are particularly useful as components in databases for sequence identity and other search analyses. As used herein, storage of the sequence information in a computer readable medium and use in sequence

databases in relation to polynucleotide or polynucleotide sequence of the invention covers any detectable chemical or physical characteristic of a polynucleotide of the invention that may be reduced to, converted into or stored in a tangible medium, such as a computer disk, preferably in a computer readable form. For example, chromatographic scan data or peak data, photographic scan or peak data, mass spectrographic data, sequence gel (or other) data.

A computer based method is also provided for performing sequence identification, said method comprising the steps of providing a polynucleotide sequence comprising a polymorphism of the invention in a computer readable medium and comparing said polymorphism containing polynucleotide sequence to at least one other polynucleotide or polypeptide sequence to identify identity (homology) i.e. screen for the presence of the polymorphism.

The invention further provides a fungal cytochrome *b* protein which confers fungal resistance to a strobilurin analogue or a compound within the same cross resistance group wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said sequence is not the *Mycena galopoda* cytochrome *b* sequence.

In a preferred embodiment of this aspect the invention further provides a fungal cytochrome *b* protein which confers fungal resistance to a strobilurin analogue or a compound within the same cross resistance group wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said sequence is not the *Mycena galopoda* cytochrome *b* sequence.

base EMBL Acession No. X87997.

The glycine residue in the protein according to the above aspect of the invention is preferably replaced by an alternative amino acid and said replacement results in the said fungi showing resistance to a strobilurin analogue or any other compound in the same cross resistance group.

The mutation according to the above aspect of the invention preferably results in the replacement of said glycine residue with an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid and most preferably alanine.

In a further aspect the invention provides an antibody capable of recognising said mutant cytochrome *b* protein.

In a further aspect the invention provides a method for the detection of a mutation in fungal cytochrome *b* gene resulting in replacement in the encoded protein of a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 said method comprising identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

In a further preferred embodiment of this aspect the invention provides a method for the detection of a mutation in fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

In a further preferred embodiment of this aspect the invention provides a method for the detection of a guanine to cytosine mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising

identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

As used herein the term around 30 means that the sequence may comprise up to 30 nucleotides, for example 5, up to 10, 15, 20, or 25 nucleotides or may comprise more than 30 nucleotides.

The test sample of nucleic acid is conveniently a total DNA preparation from fungal material, a cDNA preparation from fungal material or the fungal material itself or plant or seed extracts containing fungal nucleic acid. In this invention, we describe the detection of the $G_{143}A$ mutation by using total DNA preparation, cDNA preparation and by directly using fungal spore material as template in the PCR reactions. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample. That is to say that all or a part of the region in the sample nucleic acid may firstly be isolated or amplified using any convenient technique such as PCR before use in the method of the invention.

The present invention provides a means of analysing mutations in the DNA of agricultural field samples which by their very origin are considerably less well defined compared with an analogous situation involving human samples. Agricultural field samples are considerably more difficult to work with and it is more technically demanding to detect a mutation event occurring at a low frequency in amongst a very large amount of wild type DNA and/or extraneous DNA from other organisms present in a field isolate compared with a human sample which generally contains DNA from only one individual.

Any convenient enzyme for polymerisation may be used provided that it has no intrinsic ability to discriminate between normal and mutant template sequences to

polymerase, particularly "Ampli Taq Gold" ™ DNA polymerase (PE Applied Biosystems), Stoffel fragment, or other appropriately N-terminal deleted

modifications of Taq (*Thermus aquaticus*) or Tth (*Thermus thermophilus*) DNA polymerases.

In a further aspect the invention provides an allele specific oligonucleotide capable of binding to all or part of a fungal DNA sequence encoding a wild type cytochrome *b* protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea ,Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said oligonucleotide comprises a sequence which recognises a DNA sequence encoding a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention the said fungal DNA sequence is selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

In a further aspect of the invention we provide an allele specific oligonucleotide capable of binding to a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein said oligonucleotide comprises a sequence which recognises a DNA sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid , tryptophan, and most preferably alanine at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention we provide an allele specific oligonucleotide capable of binding to a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein selected from the group consisting of

Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea ,Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said oligonucleotide comprises a sequence which recognises a DNA sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan, and most preferably alanine at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

In a further aspect the invention provides an allele specific oligonucleotide probe capable of detecting a fungal cytochrome *b* gene polymorphism at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein.

The position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein is preferably as defined by the positions in EMBL ACCESSION NO. X84042.

In a further preferred embodiment of this aspect the invention provides an allele specific oligonucleotide probe capable of detecting a fungal cytochrome *b* gene polymorphism at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein.

The position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein is preferably as defined by the positions in EMBL ACCESSION NO. X84042.

In further preferred embodiments of the above aspect of the invention said

tritici hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis var. *difformis*

Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea ,Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.

The allele-specific oligonucleotide probe is preferably 17 to 50 nucleotides long, more preferable about 17-35 nucleotides long and most preferable about 17-30 nucleotides long.

The design of such probes will be apparent to the molecular biologist of ordinary skill. Such probes are of any convenient length such as up to 50 bases, up to 40 bases, more conveniently up to 30 bases in length, such as for example 8-25 or 8-15 bases in length. In general such probes will comprise base sequences entirely complementary to the corresponding wild type or variant locus in the gene. However, if required one or more mismatches may be introduced, provided that the discriminatory power of the oligonucleotide probe is not unduly affected. The probes of the invention may carry one or more labels to facilitate detection.

The invention further provides nucleotide primers which can detect the nucleotide polymorphisms according to the invention.

According to another aspect of the invention there is provided an allele specific primer capable of detecting a cytochrome *b* gene polymorphism at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein.

According to a preferred embodiment of this aspect of the invention there is provided an allele specific primer capable of detecting a cytochrome *b* gene polymorphism at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein.

In the above aspects the said mutation in the DNA sequence is preferably a guanine to cytosine base change.

In a further aspect the invention provides an allele specific primer capable of detecting a fungal DNA sequence encoding a wild type cytochrome *b*

protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea , Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said primer is capable of detecting a DNA sequence encoding a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention the said fungal DNA sequence is selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

In a further aspect of the invention we provide an allele specific primer capable of detecting a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein said allele specific primer is capable of detecting a DNA sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan, and most preferably alanine at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention we provide an allele specific primer capable of detecting a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea , Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said primer is capable of detecting a DNA sequence encoding a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola wherein said primer is capable of detecting a DNA sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan, and most preferably alanine at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

An allele specific primer is used generally with a common primer in an amplification reaction such as a PCR reaction which provides the discrimination between alleles through selective amplification of one allele at a particular sequence position e.g as used in the ARMS assay.

We have now devised primers for the $G_{143}A$ mutation in the above-listed fungal species which have been shown to detect the specific mutations reliably and robustly. The primers detect the guanine to cytosine base change at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein . The allele specific primers are herein referred to as diagnostic primers. In a further aspect the invention therefore provides a diagnostic primer capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and the presence of said nucleotide gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group.

The diagnostic primer of the invention is preferably at least 20 nucleotides in length, most preferably 26 nucleotides in length but this may be between 15 and 20 nucleotides in length.

In a preferred embodiment of the above aspect of the invention the penultimate nucleotide (-2) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

In a further preferred embodiment it is the -3 nucleotide of the primer which is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

Other destabilising components may be incorporated along with the -2 or -3 nucleotide.

In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied with respect to the wild type sequence without significantly affecting the properties of the diagnostic primer.

In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers comprising the sequences given below and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given below and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer.

Conveniently, the sequence of the diagnostic primer is exactly as provided below. It is preferred that the ARMS primers in all aspects of the invention are 26 nucleotides in length. In the majority of the primers listed below the penultimate nucleotide has been altered from wild type cyt *b* sequence to destabilise the primer thereby making it more selective for the desired template and these primers are particularly preferred according to the invention. It will be apparent to the man skilled in the art of primer design that bases alternative to or in addition to those discussed above may also be varied without adversely affecting the ability of the primer to bind to the template.

Primer #	species:	primer sequence for the detection of $G_{143}A$ (5' to 3')
1	*Plasmopara viticola*	CCTTGGTGACAAATGAGTTTTTGGAC
2	*Erysiphe graminis f.sp. tritici/hordei*	CCATACGGGCAGATGAGCCACTGGAC
3	*Rhynchosporium secalis*	CCTTATGGACAGATGTCTTTATGATC
4	*Pyrenophora teres*	CCCTACGGGCAAATGAGCCTTTGCGC
	Sphaerotheca fuliginea	CCCTTCGGTCAAATGTCGCTCTGGAC
8	*Uncinula necator*	CCCTACGGGCAGATGAGCCTATGGTC

9	*Colletotrichum graminicola*	CCTTACGGACAAATGTCATTATGAAC
10	*Pythium aphanidermatum*	CCTTGGTGTCAAATGAGTTTTTGGAC
11	*Colletotrichum gloeosporioides*	CCTTATGGACAAATGTCATTATGAAC
12	*Oidium lycopersicum*	CCCTACGGGCAGATGAGCCTGTGGAC
13	*Leveillula taurica*	CCATACGGACAAATGTCATTATGAAC
14	*Pseudoperonospora cubensis*	CCTTGGGGACAAATGAGTTTTTGGAC
15	*Alternaria solani*	CCTTATGGGCAAATGTCTTTATGAAC
16	*Cercospora arachidola*	CCTTATGGACAAATGTCATTATGAAC
17	*Rhizoctonia solani*	CCATACGGGCAAATGTCTCTGTGGAC
18	*Venturia inaequalis*	GTGTATGGTCAAATGAGCCTATGGCC
19	*Magnophorthe grisea*	CCTTATGGACAGATGTCATTATGAAC
20	*Phytophthora infestans*	CCTTGGGGACAAATGAGTTTTTGGAC
21	*Mycosphaerella musicola*	CCTTATGGTCAAATGTCTTTATGATC

Table 4: ARMS primer design for the detection of the $G_{143}A$ mutation

For the purposes of exemplification the primers included in Table 4 include:

- ARMS primers for P.teres, and V. ineaqualis which can be used effectively either on genomic DNA preparations or biological samples including fungal isolates, fungal cultures, fungal spores or infected plant material.
- ARMS primers for S. fulginea, O. lycopersicon, L. taurica, U. necator, or R.solani which may only be effective on cDNA
- ARMS primers for P.viticola, E.graminis f.sp tritici or hordei, R. secalis, M. graminicola, M. fijiensis var.difformis, C. graminicola, P. aphanidermatum, C. gloesporoides, P. cubensis, C, arachidola, and A. solani which may be used effectively with either genomic DNA preparations, cDNA preparations, cDNA preparations or biological samples incuding fungal isolates, fungal cultures, fungal spores or infected plant material.

cDNA material is recommended for the species where the intron/exon organisation is not currently characterised around the SNP of interest.

To adapt the primers shown in the above Table for use in a standard ASPCR reaction the last base at the 3' end should correspond to the point mutation without a destabilising base introduced.

Such primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols For Oligonucleotides And Analogues: Synthesis And Properties;" Methods

In Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

It will be appreciated that extension of a diagnostic primer can be designed to indicate the absence of the mutation resulting in a $G_{143}A$ replacement in the encoded protein. The use of ARMS primers for the detection of the absence of the mutation resulting in a $G_{143}A$ replacement in the encoded protein is preferred. Primers designed for that purpose are described herein.

The detection of the wild type sequence is useful as a control in relation to the detection of the mutation and also is necessary where quantitation of wild type and mutant alleles present in a sample is desired.

In a further aspect the invention therefore provides a diagnostic primer capable of binding to a template comprising wild type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in a wild type fungal cytochrome *b* gene said wild type fungus showing sensitivity to a strobilurin analogue or any other compound in the same cross resistance group.

In a preferred embodiment of this aspect of the invention the penultimate nucleotide (-2) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

In a further preferred embodiment the -3 nucleotide of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

Other destabilising components may be incorporated along with the -2 or -3 nucleotide.

The diagnostic primer of the invention is preferably at least 20 nucleotides in length, most preferably 26 nucleotides in length but this may be between 15 and 20 nucleotides in length.In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers capable of binding to a template

cytochrome *b* and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining

nucleotides may be varied with respect to the wild type sequence without significantly affecting the properties of the diagnostic primer.

In a further particularly preferred embodiment of this aspect of the invention we provide diagnostic primers comprising the sequences given below and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given below and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer. Conveniently, the sequence of the diagnostic primer is exactly as provided below. In the majority of the primers listed below the penultimate nucleotide has been altered from wild type cytochrome *b* sequence to destabilise the primer thereby making it more selective for the desired template. It will be apparent to the man skilled in the art of primer design that bases alternative to or in addition to those discussed above may also be varied without adversely affecting the ability of the primer to bind to the template.

Primer	Species	primer sequence for the detection of WT sequence (5' to 3')
1	*Plasmopara viticola*	CCTTGGTGACAAATGAGTTTTTGGAG
2	*Erysiphe graminis f.sp. tritici/hordei*	CCATACGGGCAGATGAGCCACTGGAG
3	*Rhynchosporium secalis*	CCTTATGGACAGATGTCTTTATGATG
4	*Pyrenophora teres*	CCCTACGGGCAAATGAGCCTTTGAAG
5	*Mycosphaerella graminicola*	CCTTATGGTCAAATGTCTTTATGAAG
6	*Mycosphaerella fijiensis* var.*difformis*	CCTTATGGTCAAATGTCTTTATGATG
7	*Sphaerotheca fuliginea*	CCCTTCGGTCAAATGTCGCTCTGGAG
8	*Uncinula necator*	CCCTACGGGCAGATGAGCCTATGGTG
9	*Colletotrichum graminicola*	CCTTACGGACAAATGTCATTATGAAG
10	*Pythium aphanidermatum*	CCTTGGTGTCAAATGAGTTTTTGGAG
11	*Colletotrichum gloeosporioides*	CCTTATGGACAAATGTCATTATGAAG
12	*Oidium lycopersicum*	CCCTACGGGCAGATGAGCCTGTGGAG
13	*Leveillula taurica*	CCATACGGACAAATGTCATTATGAAG
14	*Pseudoperonospora cubensis*	CCTTGGGGACAAATGAGTTTTTGGAG
15	*Alternaria solani*	CCTTATGGGCAAATGTCTTTATGAAG
16	*Cercospora arachidola*	CCTTATGGACAAATGTCATTATGAAG
17	*Rhizoctonia solani*	CCATACGGGCAAATGTCTCTGTGGAG
18	*Venturia inaequalis*	GTGTATGGTCAAATGAGCCTATGGAG
19	*Magnaporthe grisea*	CCTTATGGACAGATGTCATTATGAAG

20	*Phytophthora infestans*	CCTTGGGGACAAATGAGTTTTTGGAG
21	*Mycosphaerella musicola*	CCTTATGGTCAAATGTCTTTATGATG

Table 5: ARMS primer design for the detection of the wild type sequence

To adapt the primers shown in the above Table for use in a standard ASPCR reaction the last base at the 3' end should correspond to the wild type sequence without a destabilising base introduced.

The examples described above relate to ARMS primers based on the forward strand of DNA. The use of ARMS primers based on the forward strand of DNA is particularly preferred.

ARMS primers may also be based on the reverse strand of DNA if so desired. Such reverse strand primers are designed following the same principles above for forward strand primers namely, that the primers may be at least 20 nucleotides in length most preferably 26 nucleotides in length, but may be between 15 and 20 nucleotides in length and the final nucleotide at the 3' end of the primer matches the relevant template i.e. mutant or wild type and preferably the penultimate residue is optimally changed such that it does not match the relevant template. Additionally up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides in the primer may be varied without significantly affecting the properties of the diagnostic primer.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer referred to herein as the common primer, in one or more cycles of PCR amplification. A convenient example of this aspect is set out in European patent number EP-B1-0332435. The further amplification primer is either a forward or a reverse common primer. For each species, the primer used is as below. The Primers shown below are reverse primers.

	Species	primer sequence (5' to 3')
1	*Plasmopara viticola*	GATACCTAATGGATTATTTGAACCTACCT
2	*Erysiphe graminis f.sp. tritici/hordei*	AACACCTAAAGGATTACCAGATCCTGCAC
3	*Rhynchosporium secalis*	TACACCTAAAGGATTACCTGACCCTGCAC
4	*Pyrenophora teres*	TTCAAGTACATCCAATTTCAATATACACT
7	*Sphaerotheca fuliginea*	TAACTGAGAAACCCCCTCAGAGAAACTCCACAATATCTTG
8	*Uncinula necator*	TTACAGAAAAACCACCTCAAAGAAACTCCACGATATCTTG

9	*Colletotrichum graminicola*	TAACTGAGAAACCTCCTCAAACGAATTCAACAATATCTTG
10	*Pythium aphanidermatum*	CTACAGCAAATCCCCCCCATAACCAATCAACAATATCTTT
11	*Colletotrichum gloeosporioides*	TAACAGAGAAACCTCCTCAAACGAATTCAACTATATCTTG
12	*Oidium lycopersicum*	TTACAGAAAAACCTCCTCAAAGAAACTCCACGATATCTTG
13	*Leveillula taurica*	TTACAGAGAAACCTCCTCAAATAAATTCAACTATATCTTG
14	*Psuedoperonospora cubensis*	CTACAGCAAAACCGCCCCACAACCAATCAACAATATCTTT
15	*Alternaria solani*	TAACACTGAAACCTCCTCAAATGAACTCAACAATATCTTG
16	*Cercospora arachidola*	AAACAGAGAAACCTCCTCATATAAATTCAACTAAATCTTG
17	*Rhizoctonia solani*	ACACGGAAAAGCCACCCCAGATTAACTCTACAAAATCTTG
18	*Venturia inaequalis*	ATTGACTTAAGCCTCCCCACAGAAATTCGACTATATCTTG
19	*Magnaporthe grisea*	TAACAGAAAAACCACCTCAAATGAATTCAACAATATCTTG
20	*Phytophthora infestans*	CAACAGCAAAACCTCCCCATAACCAATCAACAATATCTTT
21	*Mycosphaerella musicola*	TAACAGAAAACCCACCTCAAATAAATTCAACTATATCTTG

Table 6: Examples of common primers to use with ARMS primers

In the case of the longer sequences provided in Table 6 the skilled man will be able to use this information to design appropriate primers.

It will be evident to the man skilled in the art that the common primer can be any convenient pathogen specific sequence which recognises the complementary strand of the cytochrome *b* gene (or other gene of interest) lying 3' of the mutation selective primer.

The PCR amplicon size is preferentially 50 to 400bp long but can be from 30 to 2500bp long, or potentially from 30 to 10,000bp long.

A convenient control primer may be used which is designed upstream from the $G_{143}A$ position. It will be evident to the man skilled in the art that the control primer may be any primer which is not specific for the amplification of the mutation or wild type sequences. When using these primers along with the corresponding reverse ('common') primer described above, amplification will occur regardless whether the $G_{143}A$ point mutation is present or not.

Primer	Species	Control primer sequence (5' to 3')
1	*Plasmopara viticola*	GCCTTGGGGACAAATGAGTTTTTG
2	*Erysiphe graminis f.sp. tritici/hordei*	GCCATACGGGCAGATGAGCCACTG
3	*Rhynchosporium secalis*	TCCTTATGGACAGATGTCTTTATG

4	*Pyrenophora teres*	ACCCTACGGGCAAATGAGCCTTTGAG
5	*Mycosphaerella graminicola*	TACCTTATGGTCAAATGTCTTTATGA
6	*Mycosphaerella fijiensis* var. *difformis*	GTTTTACCTTATGGTCAAATGTCTTTATG
7	*Sphaerotheca fuliginea*	TTCCCTTCGGTCAAATGTCGCTCTGG
8	*Uncinula necator*	TACCCTACGGGCAGATGAGCCTATGG
9	*Colletotrichum graminicola*	TACCTTACGGACAAATGTCATTATGA
10	*Pythium aphanidermatum*	TACCTTGGGGTCAAATGAGTTTTTGG
11	*Colletotrichum gloeosporioides*	TACCTTATGGACAAATGTCATTATGA
12	*Oidium lycopersicum*	TACCCTACGGGCAGATGAGCCTGTGG
13	*Leveillula taurica*	TACCATACGGACAAATGTCATTATGA
14	*Pseudoperonospora cubensis*	TACCTTGGGGACAAATGAGTTTTTGG
15	*Alternaria solani*	TTCCTTATGGGCAAATGTCTTTATGA
16	*Cercospora arachidola*	TACCTTATGGACAAATGTCATTATGA
17	*Rhizoctonia solani*	TTCCATACGGGCAAATGTCTCTGTGG
18	*Venturia inaequalis*	ACGTGTATGGTCAAATGAGCCTATGG
19	*Magnaporthe grisea*	TACCTTATGGACAGATGTCATTATGA
20	*Phytophthora infestans*	TACCTTGGGGACAAATGAGTTTTTGG
21	*Mycosphaerella musicola*	TACCTTATGGTCAAATGTCTTTATGA

Table 7: Examples of possible control primer design

A variety of methods may be used to detect the presence or absence of diagnostic primer extension products and/or amplification products. These will be apparent to the person skilled in the art of nucleic acid detection procedures. Preferred methods avoid the need for radiolabelled reagents. Particularly preferred detection methods are those based on fluorescence detection of the presence and/or absence of diagnostic primer extension products. Particular detection methods include gel electrophoresis analysis, "Scorpions"™ product detection as described in PCT application number PCT /GB98/03521 filed in the name of Zeneca Limited on 25 November 1998 the teachings of which are incorporated herein by reference,

patent number WO-95-21899 and surface enhanced Raman resonance spectroscopy (SERRS), outlined in patent application WO 97/05280. Further preferred detection

in published PCT application number WO 99/04037. Conveniently, real-time detection is employed. The use of "Scorpions"™ product detection as described in PCT application number PCT /GB98/03521 and published UK patent application No. GB2338301 is particularly preferred for use in all aspects of the invention described herein. The combination of the ARMS and the Scorpion technology as described herein is particularly preferred for use in all aspects of the invention described herein and the preferred detection method is a fluorescence based detection method. Many of these detection methods are appropriate for quantitative work using all of the above primers. These different PCR reactions can be carried out in different tubes or multiplexed in one tube. Using such methods, estimates can be made on the frequency of point mutation molecules present in a background of wild type molecules.

As exemplified herein we have used ARMS primers based on the forward strand of DNA in combination with Scorpion detection based on the reverse strand of DNA as the detection method. The Scorpion detection element preferably comprise the reverse primers shown in Table 6. It will be readily apparent to the man skilled in the art that alternative combinations of ARMS primers and Scorpion detection elements could also be used. For example the primer based on the forward strand of the DNA could be a combination of an ARMS primer with a Scorpion detection system and this could be used with a common primer based on the reverse strand of DNA or the primer based on the reverse strand of DNA could be a combination of an ARMS primer with a Scorpion detection system and this could be used with a common primer based on the forward strand of DNA.

In the examples described herein, the Scorpion detection element is on the common primer. The ARMS primer specific to the mutation and the wild type sequence are used in combination with the common fluorescence labelled primer. These two reactions are carried out in different PCR tubes and the fluorescence is emitted when the probe binds to the amplicon generated. The Scorpion element may alternatively be incorporated on the ARMS primers. In this case, the two ARMS primers can be labelled with different fluorophores and used along with the common primer (this time unlabelled). These three primers may be included in the same reaction as the resulting mutant and wild type amplicons will lead to different fluorescence being emitted.

As described in published UK patent application No. GB2338301 the Scorpions technology may be used in a number of different ways such as the intercalation embodiment where the tail of the Scorpions primer carries an intercalating dye which is capable of being incorporated between the bases of a double stranded nucleic acid molecule, upon which it becomes highly fluorescent; the FRET embodiment where the dyes involved in the primer form an energy transfer pair; the no-quencher embodiment where a fluorophore is attached to the tail of the Scorpions primer; the Bimolecular embodiment where the fluorophore and quencher may be introduced on two separate but complementary molecules; the Capture Probe embodiment where amplicons may be specifically captured and probed using the same non-amplifiable tail and the Stem embodiment where the primer tail comprises self complementary stems. These embodiments are described fully in published UK patent application No. GB2338301, the teaching of which is incorporated herein by reference.

The methods of the invention described herein reliably detect a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene at a detection level in the range of 1 mutated allele per 1, 000,000 wild type alleles to 1 mutated allele per 10,000 wild type alleles, and preferably in the range of 1 mutated allele per 100,000 wild type alleles to 1 mutated allele per 10,000 wild type alleles. The method of the invention can also detect mutations occurring at higher frequency, for example, 1 mutated allele per 100 wild type alleles, 1 mutated allele per 10 wild type alleles or where only mutated alleles are present. Similarly the methods of the invention may be used to detect the frequency of the wild type allele in a background of mutated alleles.

The combination of allele specific primer extension made more sensitive with use of the ARMS technology and quantitative detection methods which are used in the present invention make this an extremely valuable technique for the detection of

phenotypic bioassays to be related to the DNA profile of the target gene. The discovery of a single point mutation as the resistance mechanism explains the

qualitative nature of the resistance, and the confirmation of single spore isolate sequences validates the accuracy of the screens in determining frequencies of resistant and sensitive isolates in the samples tested.

The development of the method combining allele specific primer extension, the specificity of ARMS and real time fluorescent detection as exemplified herein with the Scorpion system enables a greater number of samples to be analysed for the presence of the resistance mutation than would be feasible in a bioassay programme. Larger sample numbers enable the identification of the resistance mutation at frequencies of a lower percentage than might be easily detected through bioassay. This enables resistance to be identified in the population before it might be apparent from field data. The high throughput nature of the method enables a wider area and more sites to be sampled and tested than might be possible using the bioassay. Allele specific primer extension such as ARMS linked with real time fluorescent detection allows the detection of the presence of the resistance gene in a population before the effects of the gene can be viewed phenotypically by bioassay in heteroplasmic and/or heterokaryotic cells, thus reducing the error of classifying samples as sensitive when they carry a low frequency of the resistance genotype. Results are obtained much faster through simultaneous read-out (real time) compared to waiting for disease development *in planta,* enabling fast responses to field situations and advice on resistance management to be given more quickly.

One or more of the diagnostic primers of the invention may be conveniently packaged with instructions for use in the method of the invention and appropriate packaging and sold as a kit. The kits will conveniently include one or more of the following: diagnostic, wild type, control and common oligonucleotide primers: appropriate nucleotide triphosphates, for example dATP, dCTP, dGTP, dTTP, a suitable polymerase as previously described, and a buffer solution.

In a further aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said

mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

In a further embodiment of this aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene.

In a preferred embodiment of this aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation, the presence of which gives rise to resistance to said fungicide, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

In a further preferred embodiment of this aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation, the presence of which gives rise to resistance to said fungicide, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the

quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene and

method comprising detecting the presence or absence of a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying and quantifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

In a further embodiment of this aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with appropriate primers in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to both the presence and absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene, and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of an amplicon generated during the PCR reaction.

In a preferred embodiment of this aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid, the presence of which gives rise to resistance to said fungicide, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a further preferred embodiment of this aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant

pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid, the presence of which gives rise to resistance to said fungicide, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended only when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a yet further aspect the invention provides a method of selecting an active fungicide and optimal application levels thereof for application to a crop comprising analysing a sample of a fungus capable of infecting said crop and detecting and/or quantifying the presence and/or absence of a mutation in a gene from said fungus wherein the presence of said mutation may give rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene and then selecting an active fungicide and optimal application levels thereof.

In a particularly preferred embodiment of this aspect of the invention the detection method comprises any (or a) single nucleotide polymorphism detection technique and is more preferably comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product and the quantification is achieved by contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to resistance to a fungicide whose target protein is

relating to the absence and the presence of the specific mutation are extended when the appropriate fungal template is present in the sample; and detecting and

the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a further particularly preferred embodiment of this aspect of the invention the detection method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product and the quantification is achieved by contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended only when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a still further aspect the invention provides a method of controlling fungal infection of a crop comprising applying a fungicide to the crop wherein said fungicide is selected according to any of the selection methods of the invention described above.

In a yet further aspect the invention provides an assay for the detection of fungicidally active compounds comprising screening the compounds against strains of fungi which have been tested for the presence or absence of a mutation giving rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene according to the methods of the invention described herein and then determining fungicidal activity against said strains of fungi.

The methods of the invention described herein are especially suitable for use with plant pathogenic fungal strains where the presence of a mutation in a cytochrome *b* gene gives rise to fungicide resistance and most especially to resistance to a strobilurin analogue or a compound in the same cross resistance group and most especially where the mutation in the fungal DNA gives rise to a replacement of a

glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143, more especially to a $G_{143}A$ replacement in the encoded protein, and especially where the mutation is a G to C base change at the second position in the codon at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

By applying the methods of the invention described herein the appropriate rate of application of fungicides and/or the appropriate combination of fungicides to be applied to the crop may be determined.

The methods of the invention described herein are particularly suitable for monitoring fungal resistance to a strobilurin analogue or a compound in the same cross resistance group in crops such as cereals, fruit and vegetables such as canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion, vines and turf.

The methods of the invention described herein are particularly sensitive at detecting low frequency of mutations in mitochondrially encoded genes, such as the cytochrome *b* gene, making this an especially useful and commercially important way of screening plant pathogenic fungi for the onset of fungicidal resistance wherein the resistance is due to a mutation in a mitochondrially encoded gene.

The invention will now be illustrated with reference to the following non-limiting Examples and Figures in which :

Figure 1 shows: a diagrammatic representation of the secondary structure of the Scorpion *P. viticola* primer

Figure 2a shows: a graph illustrating the PCR amplification of a serial dilution of *P.viticola* mutant DNA within a background of wild type DNA using the C specific primer and the Scorpion primer

Figure 2b shows: a graph illustrating a multiplex experiment with *P. viticola* ARMS primers on two plasmid dilutions

Figure 3b shows: a graph illustrating the amplification of a sensitive *E.graminis* isolate with the three primer pairs (in duplicate)

Figure 4 shows: a graph illustrating the amplification of a resistant *E.graminis* isolate with the three primer pairs (in duplicate)

Figure 5a shows: a graph illustrating the PCR amplification of a serial dilution of the wild type *R.secalis* plasmid amplified with the wild type specific primer pair

Figure 5b shows: a graph illustrating the PCR amplification of the highest concentration of the wild type and mutant *R.secalis* plasmids amplified with the wild type specific primer pair

Figure 6a shows: a graph illustrating the PCR amplification of a serial dilution of the mutant *R.secalis* plasmid amplified with the mutant specific primer pair

Figure 6b shows: a graph illustrating the PCR amplification of the highest concentration of the wild type and mutant *R.secalis* plasmids amplified with the mutant specific primer pair

Figures 7a, b and c show: graphs illustrating the PCR amplification of the amplification of the *R.secalis* DNA and cDNA templates in three dilutions with the G primer pair (in duplicate)

Figures 8 a, b and c show: graphs illustrating the PCR amplification of the amplification of the *R.secalis* DNA and cDNA templates in three dilutions with the C primer pair (in duplicate)

Figure 9a shows: a plan describing the preparation of *Pyrenophora teres* isolate K1916 for the ARMS assay

Figure 9b shows: a plan describing the preparation of *P.teres* isolates for the ARMS assay

Figure 10a and b show: graphs illustrating the amplification of the *Pteres* P13 and P15 isolates in two dilutions with the three primer pairs (in duplicate)

EXAMPLES:

In the first four Examples below the Scorpion system™ (Zeneca Diagnostics) was used as a product detection system. This detection system is described in full in PCT application number PCT/GB98/03521 filed in the name of Zeneca Limited on 25 November 1998 the teachings of which are incorporated herein by reference. This novel detection system uses a tailed primer and an integrated signalling system. The primer has a template binding region and a tail comprising a linker and a target

binding region. In use the target binding region in the tail hybridises to complementary sequence in an extension product of the primer. This target specific hybridisation event is coupled to a signalling system wherein hybridisation leads to a detectable change. The detection method of this system offers a number of significant advantages over other systems. Only a single primer/detector species is required. This provides both increased simplicity and enhanced specificity based on the ready availability of the target binding region for hybridisation with the primer extension product. The newly synthesised primer extension product is the target species so the output signal obtainable is directly related to amount of extended primer. It is not dependent on additional hybridisation events or enzymatic steps. Intra and Inter-strand competition for the probe site is limited so probe design becomes simplified. As the interaction is unimolecular, the signalling reaction is very rapid, permitting increased cycling rates which is a significant feature for primer design.

The Scorpion primers designed in the examples described below had the following modifications in common:

- A hexethylene glycerol (HEG) monomer as a blocking moiety that is sited between the template binding region of the primer and the tail region, which moiety prevents polymerase mediated chain copying of the tail region of the primer template.
- A FAM fluorescent molecule is added to the 5'end of the primer. FAM is one of the fluorescence molecules that can, for example, be readily detected by the 488nm laser of the ABI PRISM 7700
- MR is a non-fluorogenic quencher attached to a uracil

Other fluorescence molecules and quenching mechanisms are also suitable in Scorpion primer design and would be suitable to use in this invention.

In the fourth Example, an intercalating dye was used as a detection mechanism instead of the Scorpion method.

The eighteen examples in part describe the characterisation of partial

are resistant to strobilurin analogues and other compounds in the same cross resistance group.

EXAMPLE 1

In Example 1, we describe the characterisation of partial *Plasmopara viticola* cytochrome *b* gene sequence, the characterisation of a single nucleotide polymorphism (SNP) that gives rise to strobilurin analogue resistance in *P. viticola* and the validation of a real time PCR Scorpion assay for the monitoring of this SNP in *P. viticola*. A multiplexing approach to carrying out the real time PCR assay is also described. *P. viticola* is the causal agent of vine powdery mildew.

The wild type ES2B Strobilurin analogue 1 sensitive isolate of *P. viticola* was collected in 1996 from Spain. This isolate had never been exposed to strobilurin analogue selection. Infected vine leaves were hand picked and stored in a polythene bag and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival the leaves were placed in pairs, with abaxial sporulating surfaces together, over moist absorbent paper in plastic boxes, and incubated for 24-48 hours at 21-24°C. Single lesions were excised from the leaves and the sporangia suspended in 5mls deionised water, then sprayed onto the abaxial surfaces of 5-6 week old vine seedlings (var. Ohanez). Freshly inoculated plants were incubated for 24 hours in a humidity chamber (temperature ambient, relative humidity 100%), then moved to a growth room (day 24°C/r.h. 60%; night 18°C/r.h. 95%; daylength 16 hours; 6,000 lux). Plants were returned to the humidity chamber after 6 days for a further 24 hour period, after which time successful infection showed as sporulating lesions on the abaxial leaf surfaces. Further subculturing was carried out as above, adjusting the sporangial suspension to 5-10,000 sporangia per ml.

A DNA fragment encoding a significant part of wild type *P. viticola* cytochrome *b* sequence was amplified using primers based on conserved regions between *Phytophthora megasperma* and *Aspergillus nidulans* sequences (Cytb12F (5' TGAACATATTATGAGAGATGT 3') and Cyt10R (5' AATTGCATAAAAAGGTAAAAA delineating the sequence encoding amino acid region 64 and 281 based on the *S.cerevisiae* numbering system). DNA was extracted from the strobilurin analogue-sensitive isolate, using a phenol/chloroform extraction protocol. Sporangia were washed into 30mls of double distilled H_2O (ddH_2O) from six leaves with 90-100% disease cover (originated from artificially inoculated six week

old vine seedlings). The sporangial suspension was filtered through Miracloth (Calbiochem cat # 475855) and centrifuged at 3600rpm for 10mins at 4°C. The sporangia were then frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar which were previously sterilised by acid washing and autoclaving. 0.5mls of lysis buffer (200mM Tris-HCl (pH8.5), 250mM NaCl, 25mM EDTA and 0.5% SDS) was added and the material was transferred to a sterile screw cap 1.5ml Eppendorf tube. 0.5mls of a phenol/chloroform/isoamyl alcohol (25:24:1) mixture was immediately added and mixed by inverting several times. The tubes were centrifuged for 30 minutes at 14000rpm and the aqueous phase transferred to fresh Eppendorf tubes. The phenol/chloroform/isoamyl alcohol extraction was then repeated but this time the tubes were centrifuged at 14000rpm for only 15mins. After transferring the aqueous phase to a new Eppendorf tube, a final chloroform extraction was performed. The DNA was precipitated by adding 0.1 vol of 3M sodium acetate (pH5.5) and 0.6 vol of isopropanol. After inverting several times, the tubes were centrifuged at 14000rpm for 20mins at 4°C. The DNA pellet was then washed twice with 70% ethanol, vacuum dried and resuspended in 50µls of ddH_2O. The DNA yield was confirmed by gel electrophoresis and a serial dilution of the DNA (1:10, 1:100 and 1:1000) was made in ddH_2O for the use as template material in subsequent PCR reactions. PCR reactions were set up as recommended by the manufacturer of the Taq Polymerase enzyme (Gibco) and the primers were added to the reactions to a final concentration of 1pmole/µl . 10µls of each DNA dilution was added to the appropriate PCR reactions. Standard procedures were carried out in order to limit the risk of PCR contamination. 30 cycles of 94°C for 45sec, 42°C for 45sec and 72°C for 1min30sec were carried out. A final extension at 72°C for 10mins was also performed (Hybaid Omn-E PCR instrument). The efficiency of the PCR reactions was then assessed by analysing 18µls of the PCR reactions by gel electrophoresis. A 2µl sample of the PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed in *Escherichia coli* cells (TOP10 One Shot™ competent cells)(as per the

restriction digest analysis using *EcoRI*. 6 clones with suitable inserts were then

When the sequence data was analysed with the relevant bioinformatics software (Seqman, Editseq and Macaw), the resulting novel sequence encoded a new cytochrome *b* gene with close homology to other known oomycete cytochrome *b* sequences known like *P. megasperma*. The sequence of a 61 nucleotide tract *of P. viticola* encoding 30bp upstream and downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

The *P. viticola* specific primers used in later amplifications of the cytochrome *b* region of interest were PLAS17F 5' AAATAACGGTTGGTTAATTCG 3' and PLAS15R 5' TCTTAAAATTGCATAAAAAGG 3' (delineating amino acid region 73-283 according to the *S.cerevisiae* numbering system).

A strobilurin analogue-resistant isolate of *P. viticola* was identifued at one trial site. Infected vine leaves were collected and processed as above but samples were subcultured as mass populations and not isolated as single lesions prior to testing. The test method to verify strobilurin analogue resistance was a 24 hour preventative spray onto 4-week old vine seedlings. A chemical dilution series was prepared by dissolving 5mgs strobilurin analogue 1 (strobilurin analogue 1 as used in all examples herein denotes azoxystrobin) (technical material, 97% pure) in 1ml acetone and carrying out a further dilution with deionised water at room temperature to give a rate of 10ppm (a dose known to give 100% control of strobilurin analogue sensitive baseline isolate). The abaxial surfaces of the target leaves were sprayed using a DeVilbiss spray gun, 10psi, to maximum retention. Control plants were sprayed with deionised water only. The treated plants were left to dry in a growth room (conditions as above) overnight. Inoculation of the test *P. viticola* sample was carried out at 5,000 sporangia per ml and freshly inoculated plants were incubated as described previously. 7 days following inoculation any putatively resistant growth on the treated leaves was isolated and subcultured to provide sufficient material for PCR analysis. This isolate was designated T5.

Partial cytochrome *b* gene sequence was amplified with PLAS17F and PLAS15R primers from the resistant isolate (T5). Total genomic DNA (nuclear and mitochondrial) from the isolate T5 was extracted using the phenol/chloroform extraction protocol described above. Again DNA presence and quality was checked by gel electrophoresis and the DNA was diluted 1:10 and 1:100 in ddH_2O for PCR

studies. 10µls of each DNA dilution was then added to Ready.To.Go™ Taq polymerase PCR beads (Amersham Pharmacia Biotech product number 27-9555-01) and made up to 25µls with PLAS17F and PLAS15R primer solutions, each to a final primer concentration of 1pmole/µl. Standard procedures were carried out to limit the risk of PCR contamination. 30 cycles of a PCR was then carried out at 94°C for 30sec, 52°C for 30sec and 72°C for 1min. A final extension at 72°C for 10mins was also included. These PCR reactions were performed in duplicate. After the analysis of 10µls of the PCR reactions by gel electrophoresis on an 0.8% TBE agarose gel, the resulting PCR products were pooled prior to cloning. Specifically 1µl sample of the pooled PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed into *E.coli* cells (TOP10 One Shot™ competent cells)(as per the manufacturer's recommendations). A series of resulting clones were checked for the presence of inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 10 clones with the right size inserts (~500bp) were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer).

Analysis of the sequence data using suitable bioinformatics software (Seqman, Editseq and Macaw software) revealed a G-->C point mutation in the cytochrome *b* sequence when compared to wild type sequence in all 10 cases. This DNA point mutation leads to a single glycine to alanine change at position 143 (according to the *S.cerevisiae* amino acid coding system).

Different specific ARMS *P.viticola* primers were designed around this $G_{143}A$ point mutation:

two forward ARMS primers based on the wild type sequence:

G-sp-f-1: CCTTGGTGACAAATGAGTTTTTGTGG

G-sp-f-2: CCTTGGTGACAAATGAGTTTTTGGAG

and two forward ARMS primers based on the $G_{143}A$ mutation:

C-sp-f-1: CCTTGGTGACAAATGAGTTTTTGGCC

[redacted]

ST AND: GCCTTGGGGACAAATGAGTTTTTG

In all of the above ARMS primers, the -1 base (the 3'end base of the primer sequence) corresponds to the target point mutation site. Bases presented in bold differ from the wild type *P. viticola* cytochrome *b* sequence. In all of the ARMS primers (not the control primer), the -20 base was changed from a G to a T base. This was done to disrupt the run of G bases. In the G-sp-f-2 and C-sp-f-2 primers, the -2 position was changed from a G to a A base. In the G-sp-f-1, the -3 position was changed from a G to a T base. In the C-sp-f-1 primer, the -2 primer was changed from a G to a C base. These alterations to the sequence were made to destabilise the primer and render any primer extension more specific to the corresponding template. Examples in the literature have shown that destabilising the ARMS primer decreases the risk of the primer mispriming off the wrong template (Newton et al, Nucleic Acid Research 17 (7) 2503-2516 1989).

The Scorpion™ product detection system was used in this case as a detection mechanism and the Scorpion detection system was incorporated on the reverse primer. The resulting amplicon was 234 bp with the ARMS primer, and 235 bp with the standard primer. The Scorpion primer was designed using Oligo 5 and the MFold programme (MFold predicts optimal and suboptimal secondary structures for an RNA or DNA molecule using the energy minimization method by Zucker (REF). The sequence of the *P. viticola* Scorpion primer is:

FAM-CCCGCC**GTAATTGTAGGGGCTGTACTAATAC**GGCGGG MR-HEG-*GATACCTAATGGATTATTTGAACCTACCT*

Underlined regions are the hairpin forming parts, FAM is the fluorescein dye, MR is a non-fluorogenic quencher attached to a uracil and HEG is the replication blocking hexethylene glycol monomer. The sequence in italics is the reverse primer sequence and the sequence in bold is the Scorpion sequence that binds to the extension product of the reverse primer.

The stem loop secondary structure can be visualised using the MFold program (see figure 1). It has an energy of -2.2 kcal/mol in its inert form. However in the presence of the extension product the hairpin structure is separated, as the probe sequence of the Scorpion primer binds to the extension product, with an energy of -6.1 kcal/mol. This separates the FAM dye from its quencher, thus emitting fluorescence detectable for example by a 7700 instrument. The unfolding of the Scorpion element

onto the newly synthesised strand is energetically favourable compared to the Scorpion stem loop configuration in its inert state.

All primers were synthesised by Oswel DNA service (Lab 5005, Medical and Biological Sciences building, Southampton). Before use, the primers were diluted to 5μM in a total volume of 500μls each. The primers were then further diluted to a final concentration of 500nM in the PCR reactions.

In all cases AmpliTaq Gold enzyme (Perkin-Elmer/ABI) was included in the reaction mix at 1unit/25μls reaction. The reaction mix also contained 1x buffer (10mM Tris-HCl (pH8.3), 50mM KCl, 3.5 mM $MgCl_2$, 0.01% gelatine) and 100μM dNTPs. Amplifications were performed in an ABI Prism 7700 instrument for continuous fluorescence monitoring. A preliminary cycle of 94°C for 20 min was performed followed by 50 cycles of 94°C for 45sec and 60°C for 45sec. Fluorescence was monitored during the annealing/extension stage throughout the cycles.

The primers were first validated for use in such analyses by using plasmid DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. Partial wild type cytochrome *b* gene sequence and the corresponding tract containing the $G_{143}A$ mutation were cloned in the TA Invitrogen pCR2.1 vector to be used in this validation process. The C-sp-f-2 and G-sp-f-2 primers were preferred to the C-sp-f-1 and the G-sp-1 primers as duplicate PCR reactions gave more consistent results and were slightly more specific. In all cases, plasmid was diluted in 1mg/ml bovine serum albumin (BSA).

The graph shown in Figure 2a illustrates PCR reactions where a dilution of mutant plasmid in a background of normal template was amplified using the ARMS C-sp-f-2 primer. As the C plasmid dilution reduces in the wild type plasmid background, the fluorescence detection is delayed. In all cases, the final plasmid concentration in the PCR reaction was $1x10^7$ molecules/μl. With each 10 fold dilution of the C plasmid, there is a delay of 4 cycles in the detection of fluorescence. When the C plasmid is present at only 1 in 10000 copies in wild type plasmid background it

experiment.

The different materials that could be used as starting material for such resistance monitoring assays were investigated. Three different isolates were used in this study. For each of these isolates, sporangia were collected straight off diseased field vine leaves and off artificially innoculated glasshouse-grown leaves (as described previously). In both cases the sporangia were washed off the leaves using ddH_20. The sporangia were then collected by centrifugation and maintained at -80°C until needed. The sporangial samples were then resuspended in 1ml of ddH_2O and diluted 1:10 and 1:100 in ddH_20. Real time PCR assays were carried out as described above except that bovine serum albumin (0.25µg/reaction) was also added to each PCR reaction, using 5µl of each dilution as template. Each dilution was carried out in duplicate, with C-sp-f-2, G-sp-f-2 and the control forward primer; all with the common Scorpion reverse primer. The use of sporangia collected from glasshouse-grown vine leaves as starting material gave good results with good cycle threshold values (Ct). Using sporangia collected directly from field vine leaves gave poor results so the following two approaches were followed in order to improve the quality of the data: to reduce the inhibition in the PCR (field material will contain many more possible contaminants compared to glasshouse-grown leaves) and to make the DNA more available for amplification.

To reduce the effect of inhibitory components on the PCR, the concentration of BSA added to the reactions was increased and the detergent Tween-20 was added to the PCR reaction. To make the DNA more available for amplification, an initial PCR was carried out and the PCR product from this reaction was used as template in the real time PCR, the spore dilutions were boiled for 10 minutes before being added to the real time PCR to try to improve cell lysis and the DNA was extracted from the spores using 3 different protocols (DNA isolator from Genosys Biotechnologies Inc., DNAzol from Helena Biosciences, and Qiagen DNeasy plant mini kit). Each method was tried in turn with sporangia or DNA (depending on which was relevant) diluted 1:10, 1:100 and 1:1000 in ddH_2O as template. Each dilution was tested in triplicate with the C-sp-f-2 and G-sp-f-2 ARMS primers and the control primer; all with the reverse Scorpion common primer. 5µls of each template was added to the PCR reactions and the conditions were as described previously but also including 5µg/reaction BSA (when the BSA concentration was not a variable). The DNA

extracted using either DNAzol or Qiagen DNA preps gave the best results. The Qiagen DNA preparation method is favoured for future use as the protocol was simpler, quicker and therefore more adaptable to high throughput.

The G_{143} and A_{143} codon frequency was estimated in a *P. viticola* isolate collected from a field trial (denoted 17A). Sporangia were collected in ddH_20 off 20 diseased leaves and collected by centrifugation. This sample was kept at -80°C until further needed. A genomic DNA extraction using the Qiagen DNA protocol (as described above) and the resulting DNA was diluted 1:10 and 1:100 in ddH_20 for use as template for real time PCR analysis. The PCR conditions were as described above. The resulting Cycle threshold values were 20 for the G specific primer and 34 with the C specific primer giving a Ct difference of 14 cycles and thus a frequency of approximately 1 in 10000.

The Scorpion detection element was incorported on the ARMS primers to enable the G_{143} and A_{143} allele PCR amplifications to be multiplexed in the same PCR. The Scorpion/ARMS primer sequences were as follows:

G_{143} allele specific primer:

5'FAM CCCGCCC **TGGGATAGCCGAGAATAAAT** GGGCGGG MH-HEG *CCTTGGTGACAAATGAGTTTTTGGAG* 3'

A_{143} allele specific primer:

5'TET CCCGCCC **TGGGATAGCCGAGAAAAAT** GGGCGGG MH-HEG *CCTTGGTGACAAATGAGTTTTTGAGC* 3'

The Scorpion detection element details are as described above. The A_{143} allele specific primer was labelled with TET to allow the distinction to be made between the two amplicons. A reverse (unlabelled) primer was used as shown below:

Reverse 5' CATAACCAGTCAACAACTTCTTTTCC 3'

The amplicon generated in both cases was 95bp long. The Scorpion primers were designed using the Oligo5 and MFold programs. The other real time PCR components were as described above only the primer concentrations were varied during the validation of this multiplex assay. It was possible to reliably detect the ratio of 1:100 and 1:500 C:G using plasmid DNA as template (see figure 2b).

EXAMPLE 2

In Example 2, we report the characterisation of partial *Erysiphe graminis* f.sp *tritici* and *hordei* cytochrome *b* gene sequences, the characterisation of a single nucleotide polymorphism (SNP) that gives rise to resistance to strobilurin analogues or compounds in the same cross resistance group and the description of a real time PCR Scorpion assay for the monitoring of this SNP in *E. graminis* f.sp. *tritici* and *hordei*.

Isolates of *E. graminis* f.sp. *tritici* and f.sp. *hordei* (causal agents of wheat and barley powdery mildew) were collected from Northern France, Germany, Ireland and the United Kingdom. This was achieved by one of two methods: hand collection of field leaves and air spora sampling by car-mounted jet spore trap (Burkard Manufacturing Co. Ltd., Rickmansworth, UK).

Wheat leaves infected with sporulating powdery mildew were collected from sites where the populations had been exposed to strobilurin analogue 1 in previous and current field trials. Upon return to the Zeneca Agrochemicals Research centre at Jealott's Hill, the leaves were placed in polythene bags and incubated at 21°C overnight. The following day pustules were resporulating. All pustules were subcultured by tapping them above fresh leaf pieces (wheat cv Rapier, 9 days old) placed over filter paper (Whatman No. 1) in 9cm petri dishes containing 1.2% water agar. The freshly inoculated plates were incubated for 7 days and then the resulting colonies were tested for sensitivity to strobilurin analogues.

For spore trapping, wheat leaves were cut from 9 day old plants (cv. Rapier) and placed on 1.8% water agar in plastic dishes, and maintained at 5°C until required. A jet spore trap was mounted on top of a car and the car was driven at speeds up to approximately 90 km/hr along prearranged routes in each country.
The plastic dishes containing the leaf pieces were placed in the base of the spore trap column where airborne spores entering the trap settled out onto the leaves. The dishes were changed approximately every 80 km. Once a batch of leaf pieces had been used in the spore trap, they were transferred to square petri dishes containing 60mls 1.8% water agar and filter paper and stored at 5°C.

On return to Jealott's Hill the leaves exposed in the spore trap were incubated in a constant temperature room (daylength 16hrs, light 4-5,000 lux, temperature 21°C, relative humidity ambient).

5-6 days after exposure in the spore trap, *E. graminis* pustules could be seen forming (small areas of yellowing of the leaf material followed by appearance of powdery sporulating lesions). These were either subcultured onto leaf pieces in 9cm dishes as "populations" - one population per sampling stage or excised as single pustule isolates and incubated separately on leaf pieces in 5cm petri dishes on 15mls 1.2% water agar covered with filter paper. The leaf pieces inoculated as populations were incubated for 7 days after which time sporulation was sufficient to inoculate an assay. Single spore isolates were incubated for 7 days but subcultured one further time to provide enough material for testing. If sporulation was poor the above process was repeated until good (60-70%) sporulating disease coverage was obtained on all leaf pieces in order to generate sufficient conidia for an assay.

Testing and subsequent maintenance of resistant isolates was carried out on detached wheat seedling leaves treated 24 hours prior to inoculation with an aqueous solution of Srobilurin analogue 1 at 5ppm (a rate known to give 100% control of strobilurin analogue sensitive baseline isolates) and Tween 20 wetter. Isolates were tested either as mass populations or single pustule isolates. Conidia were dry-inoculated onto treated leaf pieces. Infected material was incubated in a controlled environment (as described above) for 7 days prior to assessment.

Any growth on leaf pieces treated with 5ppm Strobilurin analogue 1was considered putatively resistant. Material from these lesions was further subcultured onto strobilurin analogue 1-treated leaves to confirm resistance *in planta*, and analysed using the molecular assay described in this invention. Phenotypic resistance frequencies of approximately 1 in 100 and higher could be detected by the mass population screen, and more precise frequencies were estimated by comparing single spore isolates where growth on treated leaves was comparable to controls (resistant)

using primers based on the conserved regions of *Aspergillus niger* and *Neurospora*

ACTTAAAGGTCTAAATTG 3') delineating the sequence encoding amino acid region 93 to 323 based on the *S. cerevisiae* numbering system). Approximately 500mgs of conidia from a strobilurin analogue-sensitive isolate that had no exposure to strobilurin analogue selection were collected by tapping directly off leaves with sporulating disease into 1.5ml Eppendorf tubes. DNA was extracted from this conidial sample using a phenol/chloroform extraction protocol (see above). The DNA presence and quality was analysed by gel electrophoresis and a serial dilution of the DNA (1:10, 1:100 and 1:1000) was made in ddH_2O for use as template material in PCR reactions. PCR reactions were set up as recommended by the manufacturer of the Taq Polymerase enzyme (Gibco) and the primers were added to the reactions to a final concentration of 1pmole/µl . 10µls of each DNA dilution was added to the appropriate PCR reactions. Standard procedures were carried out in order to limit the risk of PCR contamination. 30 cycles of 94°C for 45sec, 42°C for 45sec and 72°C for 1min30sec were carried out in a Hybaid Omn-E instrument. A final extension at 72°C for 10mins was also performed. The efficiency of the PCR reactions was then assessed by analysing 18µls of the PCR reactions by gel electrophoresis. A 2µl sample of the PCR products was also cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed in *E.coli* cells (as per the manufacturer's recommendations). A series of resulting clones were checked for the presence of inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 6 clones with expected inserts size (~500bp) were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer). When the nucleotide sequence data from these studies was analysed using the relevant bioinformatics software, the resulting novel sequence was found to encode for a new cytochrome *b* gene with close homology to other known ascomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3. *E. graminis* specific primers designed based on the novel sequence were used in later amplifications of the cytochrome *b* region of interest were ERY11F 5' ATGAACAATTGGTACAGTAAT 3' and ERY12R 5' GTTAGGTATAGATCTTAATAT 3' (these delineate the sequence encoding amino acids region 114-287 according to the *S.cerevisiae* coding system)

Partial cytochrome *b* sequence was amplified with ERY11F and ERY12R primers from an initial strobilurin analogue-resistant population. Conidia (~200mg) were suspended in 200µls of ddH_2O and diluted 1:10, 1:100 and 1:1000 in ddH_2O. 10µls of each conidial dilution were added to Ready.To.Go™ Taq polymerase PCR beads (Amersham Pharmacia Biotech product number 27-9555-01) and made up to 25µls with ERY11F and ERY12R primer solutions so that the final primer concentration was 1pmole/µl. Standard procedures were carried out to limit the risk of PCR contamination. 30 cycles of a PCR reaction was carried out on a hybaid Omn-E instrument at 94°C for 45sec, 52°C for 45sec and 72°C for 1min30sec. A final extension at 72°C for 10mins was also included. All PCR reactions in this case were performed in triplicate. After the analysis of 10µls of the PCR reactions by gel electrophoresis on an 0.8% TBE agarose gel, the resulting PCR products were pooled prior to cloning. A 2µl sample of the pooled PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed in *E.coli* cells (TOP10 One Shot™ competent cells)(as per the manufacturer's recommendations). A series of clones were checked for inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 10 clones with inserts of the correct size (~500bp) were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer). Analysis of the sequence data generated using suitable bioinformatics software revealed a G-->C point mutation in the cytochrome *b* gene sequence in all 10 isolates when compared with the previously obtained wild type *E. graminis* f.sp. *tritici* cytochrome *b* gene sequence. This DNA point mutation leads to a glycine to alanine change at position 143 (according to the *S.cerevisiae* coding system).

Partial cytochrome *b* gene sequence was also characterised from two *E. graminis* f.sp. *hordei* isolates (collected in Germany). Small samples of conidia (~100mg) were tapped off infected barley leaves (which were prepared as previously

amplification. Partial cytochrome *b* gene sequences were amplified with ERY11F and

Upon gel electrophoresis analysis, a PCR product of the expected size (~500bp) was found. This product was cloned in the TA Invitrogen pCR2.1 vector and sequenced as described previously. Upon sequence analysis, it was found that the cytochrome *b* sequence amplified from *E. graminis* f.sp. *hordei* was identical to that from *E. graminis* f.sp. *tritici* apart from 1bp (T to A change) 378bp downstream of the second base of the G_{143} codon. This base does not lead to an amino acid change in the final protein.

31 different *E. graminis* isolates were studied using the above protocol and in all cases the sequence found was consistent with the $G_{143}A$ mutation being the cause for resistance to the strobilurin analogue compounds in *E. graminis* f.sp. *tritici*. The $G_{143}A$ resistance allele was not detected in any *E. graminis* f.sp. *hordei* sample.

Specific ARMS *E. graminis* primers were designed around this $G_{143}A$ point mutation:

a forward ARMS primer based on the wild type sequence:

G-sp-1: CCATACGGGCAGATGAGCCACTGG**A**G

and a forward ARMS primer based on the $G_{143}A$ mutation:

C-sp-1: CCATACGGGCAGATGAGCCACTGG**AC**

A control primer designed upstream from the point mutation:

STAND2: GCCATACGGGCAGATGAGCCACTG

In both the G-sp-1 and the C-sp-1 primers, the -1 base corresponds to the second nucleotide of the G_{143}/A_{143} codon. Bases in the primers that differ from the wild type cytochrome *b E. graminis* sequence are in bold. The -2 position was changed from a G to a A base. This was done to destabilise the primer, as is normal in ARMS reactions.

The Scorpion™ product detection system was used in this case as a detection mechanism. The Scorpion primer was designed using Oligo 5 and the MFold (see details above) programme. The sequence of the *E. graminis* Scorpion primer is:

FAM-<u>CCCGCC</u>**GTTTTAGCTGCTTTAGCTTTAATGC**<u>GGCGGG</u> MR-HEG-*AACACCTAAAGGATTACCAGATCCTGCAC*

Underlined regions are the hairpin forming parts, FAM is the fluorescein dye, MR is a non-fluorogenic quencher attached to a uracil residue and HEG is the replication blocking hexethylene glycol monomer. The sequence in italics is the reverse primer

sequence and the sequence in bold is the Scorpion sequence that binds to the extension product of the reverse primer.

All primers were synthesised by Oswel DNA service. Before use, the primers were diluted to 5μM in a total volume of 500μls each. They were then further diluted to a final concentration of 500nM in the PCR reactions.

The primers were first validated for use in ARMS/Scorpion analyses by using plasmid DNA and total DNA as templates at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. DNA fragments comprising partial wild type cytochrome *b* gene sequence and the corresponding sequence containing the $G_{143}A$ mutation were cloned in the TA Invitrogen pCR2.1 vector to be used in this validation process. The plasmid DNA was always diluted in 1mg/ml BSA prior to use as template in real time PCR assays. The DNA for analyses was extracted from a strobilurin analogue-sensitive control isolate using a phenol/chloroform extraction method (as described previously). Conidial samples from a French strobilurin analogue-sensitive isolate (F12C) and a German strobilurin analogue-resistant isolate(11-8) were then tested using the validated primers at two conidial dilutions. All three isolates originated from single pustules.

In all cases AmpliTaq Gold enzyme (Perkin-Elmer/ABI) was included in the reaction mix at 1unit/25μls reaction. The reaction mix also contained 1x buffer (10mM Tris-HCl (pH8.3), 50mM KCl, 3.5 mM MgCl2, 0.01% gelatine), 100uM dNTPs. Amplifications were performed in an ABI Prism 7700 instrument for continuous fluorescence monitoring. A preliminary cycle of 94°C for 20min was performed followed by 50 cycles of 94°C for 45sec and 60°C for 45sec. Fluorescence was monitored during the annealing/extension stage throughout the cycles.

When tested against the control templates, the *E. graminis* ARMS/Scorpion primers showed good specificity with no evidence of mispriming occurring off the wrong template. In Figure 3a, the reactions of *E.graminis* DNA at a 1:100 dilution with the three primer mixes (Stand 2 + Scorpio, G-sp-1 + Scorpio and C-sp-1 +

reaction does not show any increase in fluorescence. The control and G-specific

primer did not bind to the template present in the reaction. In this case, the genotype analysis is showing that only the wild type genotype being present.

Figure 3b illustrates the PCR reactions where the French sensitive isolate (F12C) was analysed with the three primer mixes (Stand 2 + Scorpion, G-sp-1 + Scorpion and C-sp-1 + Scorpion). In each case ~200mg of conidia were suspended in 200µls of ddH_2O and diluted 1:100 and 1:1000 in ddH_2O. 5µls of the dilutions were added to the appropriate PCR reactions. Here again, the control and G-specific primer reactions emit a good signal whilst the C-specific primer reaction does not show any increase in fluorescence. This indicates that only the wild type genotype has been detected in this sample. There is a definite delay in fluorescence being produced when using conidia as template for the reaction compared with using plasmid DNA as template. This is either due to the reduced copies of molecules that can be used as template being present in the reaction or due to inhibitory components being present in the conidial sample.

Figure 4 illustrates the PCR reactions where the German resistant isolate at two conidial dilutions was amplified using the three primer mixes (Stand 2 + Scorpion, G-sp-1 + Scorpion and C-sp-1 + Scorpion). The control and C-specific primer reactions emit a good signal whilst the G-specific primer reaction does not show any fluorescence. This indicates that only the mutant $G_{143}A$ genotype has been detected in this sample.

EXAMPLE 3

In Example 3, we report the characterisation of partial cytochrome *b* *Rhynchosporium secalis* gene sequence and a real time PCR study where various *R. secalis* isolates were screened for the $G_{143}A$ resistance allele. This is an example where the $G_{143}A$ assay was carried out on a species where the point mutation had not previously been found.

Wild type isolates of *R. secalis* were collected from the UK and France during 1981 and 1998 (see Table 6: *R. secalis* isolate details). The 1981 isolate could be considered "baseline" (collected prior to use of strobilurin analogues in the field) and 1998 isolates were obtained from Zeneca trial sites and had been exposed to several sprays of strobilurin analogues over a number of seasons. Infected barley leaves were

hand picked and stored in a polythene bag and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival at Jealott's Hill single lesions were excised from the leaves, surface sterilised in ethanol (30 seconds) followed by 0.1% sodium hypochlorite solution wash (2 minutes) then placed onto Lima Bean agar and incubated under alternating 12 hour black light/no light at a constant temperature of 19°C for 4-5 days. Colonies growing out of the lesions were subcultured as an uncounted spore suspension onto Lima Bean agar and incubated as above for approximately 7 days until sporulation was obtained. Resulting spores were removed and stored in liquid nitrogen until isolates were required. Retrieval of isolates was achieved by plating out the spore suspension onto Lima Bean agar and incubating as above for approximately 7 days until sporulation was obtained.

Isolate code	Country of origin
K1124	UK
K3327	UK
K3274	UK
K3276	UK
K3278	UK

Table 7: *R. secalis* isolate details

Partial cytochrome *b* gene sequence was obtained from two *R. secalis* isolates (K1124 and K3327). These isolates were grown from a suspension of 100,000 spores per ml inoculated in a medium with a non-fermentable carbon source shaking at 85rpm for 21 days at 19°C (12hrs light/12hrs dark) and the mycelia were collected by filtering through Miracloth and frozen at -20°C until required. DNA was produced from the mycelia by using a phenol/chloroform extraction protocol (see above). 2µls of the DNA was checked by gel electrophoresis and diluted (1:10, 1:100 and 1:1000) for use as template in PCR amplification. The PCRs were set up as described in

[illegible] that were designed in the homologous region of the cytochrome *b* fungal gene (delineating the sequence encoding amino acid

primers ERY11F and ERY12R (details as in Example 2). A band of the expected size (~500bp) was amplified using both primer pairs from each isolate and each PCR product was cloned in the Invitrogen pCR2.1 TA vector. Wizard minipreps were carried out to identify clones with inserts of the expected size (~500bp) and 5 clones were submitted for sequencing from each cloning event using the M13 forward and reverse primers. Upon analysis with the relevant bioinformatics software, it was found that a novel cytochrome *b* gene sequence had been identified which was closely related to other ascomycete cytochrome *b* gene sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

Different specific ARMS *R. secalis* primers were designed around the $G_{143}A$ point mutation location:

two forward ARMS primer based on the wild type sequence:

G-sp-2: CCTTATGGACAGATGTCTTTATGA**T**G

G-sp-3: CCTTATGGACAGATGTCTTTATGA**A**G

and two forward ARMS primer based on the $G_{143}A$ mutation location:

C-sp-2: CCTTATGGACAGATGTCTTTATGA**TC**

G-sp-3: CCTTATGGACAGATGTCTTTATGA**AC**

A control primer designed upstream from the point mutation:

STAND3: TCCTTATGGACAGATGTCTTTATG

In the ARMS primers, the -1 base (the 3'end base) corresponds to the second nucleotide of the G_{143}/A_{143} codon. Bases in the primers which differ from the wild type cytochrome *b R. secalis* sequence are in bold. The -2 position was changed from a G to an A or T base. This was done to destabilise the primer, as is normal for ARMS primers.

The Scorpion™ product detection system was used in this case as a detection mechanism and the Scorpion reverse primer was again designed using the Oligo 5 package and the MFold program. The *R. secalis* Scorpion primer sequence was:

FAM-<u>CCCGCC</u>**ATATTAGCTGCATTAGTATTAATGC**<u>GGCGGG</u>-MR-HEG-*TACACCTAAAGGATTACCTGACCCTGCAC*

(See previous examples for details).

All primers were synthesised by Oswel DNA service. Before use, the primers were diluted to 5μM in a total volume of 500μls each. The primers were then further diluted to a final concentration of 500nM in the PCRs.

Primers were first validated for use in ARMS/Scorpion analyses by using plasmid DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. Partial wild type cytochrome *b* gene sequence and a corresponding sequence containing the $G_{143}A$ mutation were cloned in the TA Invitrogen pCR2.1 vector to be used in this validation process. As this mutation has not yet been found in *R. secalis* DNA, the A_{143} point mutation was incorporated into the sequence using a PCR strategy: the point mutation was incorporated into a primer design and was used to amplify the region of interest using the wild type clone as template. PCR reactions were set up using standard methods as previously described and 30 cycles of 94°C for 45sec, 56°C for 45sec and 72°C for 1min30 were performed. A final extension incubation of 10mins at 72°C was also included. The resulting PCR product was cloned into the TA Invitrogen pCR2.1 vector and a resulting clone was sequenced to check for any PCR induced errors prior to use in this experiment.

Undiluted, the plasmid DNA preparations were calculated to be at concentrations around $2x10^{11}$ molecules per μl. The two plasmids were therefore diluted to $2x10^7$, 10^5, 10^3 and 10^1 molecules/μl in 1mg/ml BSA and 5μls were used of each dilution resulting in ~ $1x10^8$, 10^6, 10^4 and 10^2 molecules of plasmid in the respective PCR reactions. The PCR conditions and components were as described previously. In figure 5a, which shows a serial dilution of the G plasmid detected with the G primer mix, the detection of fluorescence is delayed by ~ 4 cycles with each 10 fold plasmid dilution. Figure 5b, which shows the highest concentration G (wild type (wt)) and C (mutant) cassette amplified with the G-sp-2 primer mix. The G primer does not misprime off the C template until very late in the PCR even though the DNA template concentration is high (~10^8 molecules of template in reaction). The

specificity through the specific and non specific plasmid dilutions (figure 6a and 6b).

G-sp-2 and C-sp-2 primer mixes were used in following experiments instead of G-sp-3 and C-sp-3 primer mixes.

The second part of this study was to compare using total DNA and cDNA as template for the PCR. Total DNA material was prepared from all isolates of *R.secalis* using a phenol-chloroform extraction method (as described previously). Total RNA was extracted from 100mg of ground mycelia using the RNeasy kit from Qiagen (according to the manufacturer's recommendation). First strand cDNA synthesis was then undertaken with 1μg of total RNA using RT PCR with the Advantage RT-PCR Clontech kit (according to the manufacturer's recommendation). Pools of total DNA and cDNA from three isolates (K3278, K3274 and K3276) were prepared. The total DNA pool was used as template diluted 1:100, 1:1000 and 1:10000 and the cDNA was used as template neat and diluted 1:5 and 1:10. In each case, 5μls of template was added to the PCR reactions. Real time PCR conditions described in Example 1 and 2 were also used in this case except that 40 cycles PCR were performed in this case instead of 50. Figure 7a, b and c illustrate total DNA and cDNA templates at three dilutions (dilution 1: total DNA (1:100) and cDNA (neat); dilution 2: total DNA (1:1000) and cDNA (1:5); dilution 3: total DNA (1:10000) and cDNA (1:10)) amplified using the G primer mix. Figures 8a, b and c illustrate the total DNA and cDNA templates amplified using the C primer mix. Fluorescence could be detected slightly earlier in the total DNA samples so in order to give the best chance of detecting any C mutation, total DNA inputs at dilutions of 1:10 and 1:1000 were the chosen template for future analyses. No fluorescence changes could be detected when the C specific primer mix was used in this 40 cycles PCR reaction.

EXAMPLE 4

In Example 4, we report the characterisation of partial *Pyrenophora teres* cytochrome *b* gene sequence and a study where a $G_{143}A$ resistance allele detection assay was carried out on a variety of *P. teres* isolates. This is a species where the $G_{143}A$ mutation had not previously been identified. This example is divided into two section; one describing a real time PCR study using an intercalating dye on cDNA preparations of *P. teres* isolates and the other describing a real time PCR study using a Scorpion assay on genomic DNA preparations of *P. teres* isolates.

Real time PCR study using an intercalating dye:

Wild type isolates of *P. teres* (causal agent of barley net blotch) were collected from the UK and France during 1994, 1996 and 1998 (see Table 8 for *P. teres* isolate details). 1994 and 1996 isolates could be considered "baseline" (collected prior to use of strobilurin analogues in the field) and 1998 isolates were obtained from Zeneca trial sites which had been exposed to several sprays of strobilurin analogues over a number of seasons. Infected barley leaves were hand picked and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival at Jealott's Hill the leaves were incubated in a humid environment at 21° for 24-48 hours. Single lesions were excised from the leaves, surface sterilised in ethanol (30 seconds) followed by 0.1% sodium hypochlorite solution (2 minutes) then placed onto Rose Bengal agar and incubated under alternating 12 hour black light/no light at a constant temperature of 22°C for 4-5 days. Colonies growing out of the lesions were subcultured by mycelial plug onto V8+streptomycin agar and incubated as above for 14 days. Resulting mycelial and spore material was removed and stored in liquid nitrogen until isolates were required. Retrieval of isolates was achieved by plating out stored fungal material onto V8 agar and incubating as above for 14 days until sporulation was obtained.

Isolate code	Year collected	Country of origin
K1056	1980	Ireland
K1916	1994	UK
K2346	1996	France
K2383	1996	UK
K2385	1996	UK
K2390	1996	UK
K2396	1996	UK
K3230	1998	UK
K3237	1998	UK
K3238	1998	UK
K3253	1998	UK

Table 8: *P. teres* isolate details

Partial cytochrome *b* sequence was identified from two isolates (K1056 and

The flask was incubated at 85rpm on an orbital shaker under 12 hours white light 12

mycelial material for the DNA extraction protocol. The mycelium was then harvested by filtering through Miracloth and was frozen at -20°C until needed. Total DNA was produced using the phenol/chloroform extraction protocol (see Example 1). After checking the DNA by gel electrophoresis, it was diluted (1:10, 1:100 and 1:1000) and used as a PCR template. Many different primer combinations were tried (specific and degenerates) and in most cases no amplification product was obtained. However, a primer pair that was designed to conserved *Aspergillus niger* and *Neurospora crassa* cytochrome *b* sequences (Cytb3F and Cyt9R, see Example 2 for details) did give a PCR product. This product was cloned in the TA Invitrogen pCR2.1 vector and 6 clones were sequenced using M13 forward and reverse primers. Upon analysis of the results, it was discovered that the reverse primer had correctly bound to cytochrome *b* sequence whilst the forward primer had misprimed in DNA sequence with intron-like features. A specific *P. teres* primer was designed in the novel stretch of cytochrome *b* gene and was used with a forward primer that was designed based on *Venturia inaequalis* cytochrome *b* sequence sequence? on *P. teres* cDNA template. cDNA was produced from mycelium for the two isolates. Total RNA was extracted from 100mg of ground mycelium using the RN easy kit from Qiagen (according to the manufacturer's recommendation). First strand cDNA synthesis was prepared from 1µg of total RNA using RT PCR with the Advantage RT-PCR Clontech kit (according to the manufacturer's recommendation). 5µls of the resulting cDNA was then used in PCR reactions. The PCR components and conditions were as described previously. A PCR product was amplified for both isolates (covering amino acid region 48 to 311 according to the *S.cerevisiae* coding system). In both cases, the amplicon was cloned in the TA pCR2.1 Invitrogen vector and 4 positive clones were sequenced using M13 forward and reverse primers for each isolate. Sequence data analysis revealed that a novel cytochrome *b* gene sequence had been isolated that was closely related to other known ascomycete cytochrome *b* sequences. A 61 nucleotide tract of cDNA sequence encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

Specific ARMS *P. teres* primers were designed around the $G_{143}A$ point mutation location:

A forward ARMS primer based on the wild type sequence:

G-sp-4: CCCTACGGGCAAATGAGCCTTTGAAG

A forward ARMS primer based on the $G_{143}A$ mutation location:

C-sp-5: CCCTACGGGCAAATGAGCCTTTGA**TC**

A control primer designed upstream from the point mutation:

STAND4: ACCCTACGGGCAAATGAGCCTTTG

The reverse primer used was:

UNLS4: TACACCTAAAGGATTTCCTGACCCTGCAA

In the ARMS primers, the -1 base (the 3'end base) corresponds to the second nucleotide of the G_{143}/A_{143} codon. Bases in the primers which differ from the wild type cytochrome *b P. teres* sequence are in bold. The -2 position was changed from a G to an A or T base. This was done to destabilise the primer.

All primers were synthesised by Oswel DNA service. Before use, the primers were diluted to 5uM in a total volume of ddH_2O 500µls each. The primers were then further diluted to a final concentration of 500nM in the PCR reactions.

In this case, we made use of an intercalating dye for the detection of PCR product. The dye used was YO-PRO-1 dye (Molecular Probes, Seattle Washington, USA) which binds to double stranded DNA and emits fluorescence which is detected by the ABI7700.

The primers were first validated by using plasmid DNA as template at various concentrations. This was done to check the specificity and sensitivity of the primer designs. Partial wild type *P. teres* cytochrome *b* gene sequence and a corresponding sequence containing the $G_{143}A$ mutation introduced by site directed mutagenesis were cloned in the TA Invitrogen pCR2.1 vector to be used in this validation process.

Undiluted, the plasmid DNA stocks were calculated to be at around $2x10^{11}$ molecules per µl. The two plasmids were diluted to $2x10^7$, 10^5, 10^3 and 10^1 molecules/µl and 5µls were used of each dilution resulting in ~ $1x10^8$, 10^6, 10^4 and 10^2 molecules of plasmid in the respective PCR reactions (see Example 1 for PCR

This method of detection is less sensitive than the Scorpion detection system because

formation). It was concluded that valuable information could be drawn from using this method but increased caution has to be taken in interpreting results.

In the following experiment, various different isolates were checked for the presence of $G_{143}A$ allele. The isolates tested in this example were prepared as described below:

Isolates were grown in a medium with a non-fermentable carbon source and varying concentrations of strobilurin analogue 2 to obtain material for ARMS diagnosis. (See Figure 9a: Preparation of *P. teres* isolate K1916 for the ARMS assay and Figure 9b: Preparation of *P. teres* isolates for the ARMS assay.) An initial spore suspension, where obtained, was inoculated (1ml at 100,000 spores/ml) into conical flasks containing broth amended with strobilurin analogue 2 (=picoxystrobin) (1 flask per isolate per concentration). The material was incubated on an orbital shaker as previously described. Once sufficient material was obtained mycelium was either submitted for ARMS diagnosis or further subcultured at increased rates of strobilurin analogue 2 (see Figures 9a & b) before testing.

cDNA material was prepared (as described previously) from each of the isolates described above. This was done to avoid designing primers within the complex intron/exon organisation of the *P. teres* cytochrome *b* sequence. The isolates were pooled as described below:

Isolate #	Isolate name	growth conditions
P1	K1916	0.04ppm
P2	K1916	0.16ppm
P3 - pool 1	K3238	0.01ppm
P4 - pool 1	K2346	0.01ppm
P5 - pool 1	K2396	0.01ppm
P6 - pool 1	K2390	0.01ppm
P7 - pool 2	K3230	0.02ppm
P8 - pool 2	K3237	0.02ppm
P9 - pool 3	K2383	0.01ppm
P10	K1916	0ppm
P11 - pool 3	K3253	0.01ppm
P12 - pool 3	K2385	0.01ppm
P13	K1916	0ppm
P14	K1916	30ppm
P15	K1916	100ppm

Table 9: *P. teres* isolate details

All PCR reactions were set up as previously described and 50 cycles were carried out. The only difference was that YO-PRO-1 dye was added to the reaction mix. The three primer pairs were used on the three pools and the 6 single isolates

tested. cDNA neat and diluted 1:10 were used as template, in all cases 5µls of template were added to the PCR reactions.

Figures 10 a and b illustrate the PCR amplification results of P13 and P15 isolates in two dilutions, in duplicate with the three primer pairs. This experiment shows that even though P15 was grown in 100ppm strobilurin analogue selection, genotypically it does not appear any different to P13 which was grown without strobilurin analogue selection. When the other samples were tested using this ARMS/Scorpion assay, the C primer mix did not show any fluorescence until the primer dimer formation interferes with the data.

Real time PCR study using a Scorpion detection system:

A diagnostic G143A Scorpion assay was also designed based on the genomic organisation that was determined for the *P. teres* cytochrome *b* gene around the amino acid region of interest.

The intron/exon organisation around the base of interest was elucidated by carrying out PCR amplifications on genomic DNA preparations with a series of primers designed to the known coding sequence and the short stretch of intron sequence that was found during the mispriming event descibed above. Taq Extender™ PCR Additive and Perfect Match® PCR Enhancer (both from Strategene) were used (as per manufacturer's instructions) in the amplification of large PCR fragments using a variety of different primer combinations. After an initial 94°C step for 3min, 30 cycles of 94°C for 45sec, 52°C for 45sec and 72°C for 3min were carried out on a Hybaid Omn-E PCR instrument. A final 72°C step for 10min was also included. When the PCR products were analysed using gel electrophoresis, a 2.7kb PCR product was found with the primer pair pter23F (5' ACA TAG TAA TAC TGC TTC AGC 3') - pter25R (5' TAC ATT TGA GGC AAA TAT TTC 3') and a 7.5kb PCR product was found with primer pair pter7F (5' CTA CGG GCA AAT GAG CCT TTG 3') - pter6R (5' CTC TGG AAC TAT CGC TGC AGG 3'). These PCR products

located at the 3' end of a 36bp exon. A 61 nucleotide tract encoding 30 bases

upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

A series of 31bp ARMS primers and a control primer (for the amplification of both mutant and wild type alleles) were designed on the forward strand whilst the common Scorpion primer was designed on the reverse strand in intron sequence. The resulting amplicon was 121bp long (with the ARMS primers) and 123bp long (with the control primer). In this case, the second base of the G_{143} codon is on a 37bp exon, only 1bp away from the 3' splicing site. The Scorpion primer was designed using the Oligo 5 package and the MFold program (see details in Example 1). The *P. teres* Scorpion primer sequence was:

5'FAM CCC GCC **GCA AGC TGA TTT CAT A**GG CGG G MR-HEG *TT CAA GTA CAT CCA ATT TCA ATA TAC ACT* 3'

The Scorpion primer description, the primer synthesis and real time PCR conditions were as described in previous examples.

In the optimisation of this Scorpion assay, a wider range of primers than the ones previously designed were tested for specificity, each with different mismatched bases at their 3' end to cause destabilisation (highlighted in bold), as shown below.

Primer	Sequence
STAND6	A CCC TAC GGG CAA ATG AGC CTT TGA G
PT-G-1	CCC TAC GGG CAA ATG AGC CTT TGA **A**G
PT-C-1	CCC TAC GGG CAA ATG AGC CTT TGA **A**C
PT-G-2	CCC TAC GGG CAA ATG AGC CTT TGA **C**G
PT-C-2	CCC TAC GGG CAA ATG AGC CTT TGA **C**C
PT-G-3	CCC TAC GGG CAA ATG AGC CTT **C**GA **A**G
PT-C-3	CCC TAC GGG CAA ATG AGC CTT **C**GA **A**C
PT-G-4	CCC TAC GGG CAA ATG AGC CTT T**T**A **C**G
PT-C-4	CCC TAC GGG CAA ATG AGC CTT T**T**A **C**C
PT-G-5	CCC TAC GGG CAA ATG AGC CTT TG**C** GG
PT-C-5	CCC TAC GGG CAA ATG AGC CTT TG**C** GC

Table 10

Initial validation of the primers was carried out on the wild type and mutant plasmid template at $1x10^7$ molecules per reaction. Real time PCR on the ABI PRISM 7700 instrument (PE Biosystems) was carried out in triplicate for each of the plasmid templates, using both the G and C-specific ARMS primers and the control primer

(which amplifies both mutant and wild type alleles) with the common reverse Scorpion primer.

As seen in the table below, the different ARMS primer designs gave different windows of specificity when tested against mutant and wild type plasmid templates. When compared to the other primers tested, PT-G-1 and PT-C-5 provide the widest window of specificity. They were therefore chosen as the preferred primer pair for this assay. Primer PT-C-5 has a Ct value of 16 on the correct template, and PT-G-1 also has a Ct value of 16 on the correct template. The PT-C-5 primer misprimes on the wrong template at cycle 34 giving a window of specificity of 18 cycles and the PT-G-1 primer misprimes at cycle 32 giving a window of specificity of 16 cycles equating to approximately 10 fold difference in the sensitivity of the two primers.

Primer	Ct on correct template	Ct on incorrect template	Window of specificity (cycles)
PT-G-1	16	32	16
PT-G-2	23	38	15
PT-G-3	FAILED	FAILED	FAILED
PT-G-4	32	42	10
PT-G-5	22	36	14
PT-C-1	20	34	12
PT-C-2	20	34	12
PT-C-3	28	38	10
PT-C-4	34	38	4
PT-C-5	16	34	18
C-SP-5	18	34	18

table 11:

The PT-G-1 and PT-C-5 ARMS primers were tested in a plasmid spiking experiment where the mutant plasmid was 'spiked' in a background of wild type plasmid at frequencies of 1:1, 1:10, 1:100, 1:1000, 1:10000 and 1:100000, all in 1mg/ml BSA. In all cases the total plasmid concentration of each frequency in the PCR assay was $1x10^7$ molecules/reaction. Real time PCR conditions and components were as described previously. The resulting graph can be seen in figure x. The PT-C-5 primer mispriming from the 100% wild type (G) cassette does mask the lower C:G

was not distinguishable. This confirms that the ARMS switch in this case will only allow a frequency of <1:10000 to be detected with confidence.

A series of *P. teres* samples gathered from Cork in Ireland (Ir 5-8, Ir 9-13, Ir 14-17, Ir 18-21, Ir 30-34) were tested using the newly optimised real time Scorpion PCR assay. These groups of 4 or 5 isolates were inoculated onto V8 agar plates and incubated until sporulation on the resulting colony was achieved.

Mycelia were harvested from the agar plates in 10ml sterile ddH_2O. The mycelial suspension was transferred to a 15ml falcon tube and centrifuged at 3200rpm for 10 minutes. The water was removed and the mycelial mass was divided between two sterile Eppendorf tubes. The mycelia were then pelleted by centrifugation at 13000rpm for 5 minutes. The supernatant was removed using a pipette and one of the mycelial pellets was used in the DNA extraction. DNA extraction was carried out using the Qiagen DNeasy Plant Mini Kit protocol for isolation of DNA from plant tissue, as described in the manufacturer's protocol. The DNA was diluted 10-fold and 100-fold for use in the assay and 5µl of DNA were used in each PCR assay. All isolates were tested with each primer pair (PT-C-5, PT-G-1 and the control primer; each with the common reverse primer containing the Scorpion detection system) in triplicate and in two dilutions.

The results of the isolate screening using the 1:10 dilution of genomic DNA as template are shown below:

Isolates	Cycle threshold values		
	C	G	S
Ir5-8 (1)	-	20	20
Ir5-8 (2)	-	22	22
Ir5-8 (3)	34	20	20
Ir9-13 (1)	-	18	18
Ir9-13 (2)	-	20	20
Ir9-13 (3)	36	20	20
Ir14-17 (1)	36	18	18
Ir14-17 (2)	-	22	22
Ir14-17 (3)	36	18	18
Ir18-21 (1)	-	20	20
Ir18-21 (2)	-	20	20
Ir18-21 (3)	34	18	18
Ir30-34 (1)	-	20	20
Ir30-34 (2)	34	20	20
Ir30-34 (3)	-	19	19

table 12 : *P. teres* Real Time PCR results

In most cases the PT-C-5 primer only shows a cycle threshold value (Ct) at or later than cycle 34 at which mispriming is shown to occur on plasmid template. In

some cases the PT-C-5 primer does not misprime at all. These results demonstrate that there was no evidence of the G143A mutation being present in the Ir5-8, Ir9-13, Ir14-17, Ir18-21 or Ir30-34 samples.

EXAMPLE 5

In Example 5, we report the characterisation of partial cytochrome *b Uncinula necator* gene sequence.

Infected vine leaves and fruit were collected from trial sites and commercial vineyards in France and Italy during 1999 and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Following arrival at Jealott's Hill mycelium and spores were transferred using a small paintbrush to fresh surface-sterilised leaves detached from 6-7 leaf seedlings (var. Ohanez). *U. necator* from each collection site was treated as a separate population.

On receipt in the laboratory the inoculated leaves were placed in a constant temperature room at 21°C and incubated for 2-3 weeks. Once sporulating disease was detected, the infected leaves were used to inoculate 3-4 leaf vine seedlings sown directly into a plastic plant propagator. A compressed airline was used to blow conidia off source leaves onto appropriate target leaves of up to 40 vine seedlings per isolate. The lid was replaced on the propagator, which was then incubated in a controlled environment growth room for 2 weeks. Conidia produced on these plants were inoculated onto an *in planta* sensitivity assay.

Appropriate target leaves of 3-4 leaf vine seedlings were sprayed by DeVilbiss spray gun (MP538) on the adaxial surface with a discriminating dose (10ppm, dose known to give a 100% control of sensitive wild type strains) of commercial formulation of Srobilurin analogue 1. For each population to be tested, 10 plants were treated with water only as controls and 30 plants treated with Srobilurin analogue 1. Known strobilurin analogue-sensitive baseline isolates were included in each test to check efficacy of the discriminating dose (single spore isolates collected from France

conidia were removed for PCR analysis, and the remainder suspended in

were sprayed as above. Once dry the plants were placed randomly in a plastic propagator, the lid replaced and the test incubated in a controlled environment for 2 weeks before assessment. Any growth on treated leaves was subcultured and re-tested to check for strobilurin analogue resistance.

Total RNA was extracted from 100mg of ground *U. necator* spores using the RNeasy kit from Qiagen (according to the manufacturer's recommendation). First strand cDNA synthesis was prepared from 1µg of total RNA using RT PCR with the Advantage RT-PCR Clontech kit (according to the manufacturer's recommendation). 5µls of cDNA were used as template for PCR amplification using ERY 11F (5' ATGAACAATTGGTACAGTAAT 3') and ERY 4R (5' AAATCTGTTAAAGGCATAGCC 3') which delineate amini acids 114 to 309 based on the *S. cerevisiae* numbering system). A DNA fragment of the expected size (~500bp) was amplified and the PCR product was cloned in the Invitrogen pCR2.1 TA vector (according to manufacturer's recommendations). Wizard minipreps were carried out to identify clones with suitable inserts and 5 clones were submitted for sequencing using the M13 forward and reverse primers from each cloning event. Upon analysis with the relevant bioinformatics software (Seqman and Macaw), it was found that a novel cytochrome *b* gene sequence had been identified which was closely related to other ascomycete cytochrome *b* sequences. A 61 tract of cDNA sequence encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3. Specific *U. necator* primers were designed based on the novel sequence for use directly on spore material. The presence of intron sequences within the region of interest (500bp around the G_{143} SNP) was suspected as no previous PCR amplification on biological material had been successful. Taq Extender™ PCR Additive and Perfect Match® PCR Enhancer (both from Strategene) were used (as per manufacturer's instructions) in the amplification of large PCR fragments using a variety of different primer combinations. After an initial 94°C step for 3min, 30 cycles of 94°C for 45sec, 52°C for 45sec and 72°C for 3min were carried out on a Hybaid Omn-E PCR instrument. A final 72°C step for 10min was also included. Primer combinations 2F (5' GTT TTA CCC TAC GGG CAG ATG 3')-5R(5' AAA GAA TCT GTT TAA GGT TGC 3'), 2F6R (5' AAA CCA CCT CAA AGA AAC TCC 3') and 4F (5' CAT

GAA TAG GAC AAG ATA TCG 3')-6R successfully amplified PCR products ranging from 1.6kb to 3kb in length. These PCR products were cloned in the TA Invitrogen pCR2.1 vector and subsequent clones were used for sequencing as described previously. A primer walking strategy was followed for one clone corresponding to each of the different PCR products and the sequence analysis of these three clones showed the presence of two introns (1.6kb and 1.1 kb in length) respectively 61bp upstream and 7bp downstream from the second base of the G_{143} codon. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

EXAMPLE 6:

In example 6 we report the characterisation of partial *Sphaerotheca fuliginea* cytochrome *b* gene sequence and a single nucleotide polymorphism that gives rise to resistance to strobilurin analogues and compounds in the same cross resistance group. *S. fuliginea* is the causal agent for cucurbit powdery mildew.

S. fuliginea infected cucumber and melon leaves were collected from the field and conidia dry inoculated to fresh leaf material (cucumber and melon) using a small paintbrush. Monoconidial isolates were subcultured and tested *in planta* by 24 hour preventative discriminating dose assay (up to 100ppm doses, known to give a 100% control of wild type strains). Conidia from putatively resistant isolates were removed by aspiration by vacuum pump into a suitable container. Part of this sample was analysed using a PCR analysis and the remainder was re-tested to confirm phenotypic resistance.

An RT-PCR strategy was followed (as described in Example 3) on a ~100mg conidial strobilurin analogue sensitive sample. The resulting cDNA was used as template in a PCR amplification reaction using primers Ery2F (5' TCACCTAGAACATTAACATGA 3') and 4R (5'

described previously. A PCR product of the expected size (~600bp) was found during gel electrophoresis analysis of the PCR products and this product was then cloned in

the TA Invitrogen pCR2.1 vector and 5 clones with correct size inserts (~600bp) were sequenced using M13 forward and reverse primers as described previously. Upon analysis with the relevant bioinformatics software (Seqman and Macaw), it was found that a novel cytochrome *b* gene sequence had been identified which was closely related to other ascomycete cytochrome *b* sequences. Specific *Sphaerotheca fuliginea* PCR primers were then designed for the amplification of the G_{143} region from genomic DNA. Primer pairs SF1 (5' TTCCCTTCGGTCAAATGTCGC 3') - SF8 (5' AAACCCCCTCAGAGAAACTCC 3') and SF1 - SF10 (5' GACCCCGCGCTATCATGTAAG 3') were used in PCR amplifications using spore samples resuspended in H_20 as template. The PCR components and conditions were as described in previous examples. The PCR products were analysed by gel electrophoresis and a 2kb product was found with the SF1/8 primer pair and a 2.1kb band was found with the SF1/10 primer pair. Both PCR products were cloned in the TA Invitrogen pCR2.1 cloning vector and 5 clones were sequenced as described previously. Two clones for both cloning events were fully sequenced using a primer walking strategy. When the sequence data was analysed using the relevant bioinformatics software, it was found that a 1917bp intron was present 8bp downstream from the second base of the G_{143} codon. The cDNA and the genomic (taking in account the intron/exon organisation) sequences of a 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

Partial cytochrome *b* gene sequence was also amplified from conidial strobilurin analogue resistant samples. Conidial samples (<50mg) were resuspended in 200µls ddH_20 and diluted 1:10, 1:100 in ddH_20. 5µls of each dilution was used as template for PCR amplification using the SF1/SF8 specific *S. fuliginea* primers. Taq Extender™ PCR Additive and Perfect Match® PCR Enhancer (both from Strategene) were used (as per manufacturer's instructions) in the amplification of this large PCR fragment. After an initial 94°C step for 3min, 30 cycles of 94°C for 45sec, 50°C for 45sec and 72°C for 5min were carried out on a Hybaid Omn-E PCR instrument. A final 72°C step for 10min was also included. The PCR products were analysed using gel electrophoresis analysis and an expected size band (~2kb) was found. This was

cloned in the TA Invitrogen pCR2.1 vector and 10 clones which had the correct size insert were sequenced as described in previous examples. Analysis of the sequence data using suitable bioinformatics software (Seqman, Editseq and Macaw software) revealed a G-->C point mutation in the cytochrome *b* sequence when compared to wild type sequence in all 10 cases. This DNA point mutation leads to a single glycine to alanine change at position 143 (according to the *S.cerevisiae* amino acid coding system).

EXAMPLE 7:

In example 7 we report the characterisation of partial *Mycosphaerella fijiensis* var. *difformis* cytochrome *b* gene sequence. *M. fijiensis* is the causal agent of Black Sigatoka disease on banana.

Infected banana leaves were collected from the field and ascospores inoculated from leaves directly onto artificial media in a petri dish. Monoascosporic isolates were maintained on artificial media and prepared for PCR analysis by shake flask culture in a broth medium. Mycelia were collected through Micracloth and ground to a fine powder using a sterile pestle and mortar. 100mg of ground mycelia were used in a genomic DNA extraction and in a first strand cDNA synthesis (as described in previous example). The genomic DNA was diluted 1:10, 1:100 and 1:1000 prior to use as PCR template. 5µls of cDNA and each genomic DNA dilution were used as template for PCR amplification using the degenerate primer pair described in Example 3. The PCR conditions and components were as described in previous Examples. Upon gel electrophoresis analysis of the PCR products, an expected size band (~500bp) was found when cDNA was used as template and a larger band (~1.6kb) was found when genomic DNA was used as template. The two PCR products were cloned in the Invitrogen pCR2.1 TA vector and five resulting clones with the correct size inserts (~500bp and 1.6kb) were sequenced using the M13 forward and reverse primers. Upon analysis of the sequence data using the [illegible]

presence of the intron in the larger PCR product, the DNA sequences were identical.

other ascomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S. cerevisiae* amino acid numbering system) can be found in table 3. *Mycosphaerella musicola*?

EXAMPLE 8:

In example 8 we report the characterisation of partial *Pseudoperonospora cubensis* cytochrome *b* gene sequence. *P. cubensis* is the causal agent of cucurbit downey mildew.

Sporangia were washed off infected leaf material into deionised water. The resulting sporangial suspension was inoculated by spray gun onto fresh leaf material at a concentration of 10,000 spores per ml. A sample of spore suspension was spun in a centrifuge to produce a pellet and kept at -80°C until further needed. The *P. cubensis* sporangia were then resuspended in 200µls of ddH_20 and diluted 1:10 and 1:100 in ddH_20. 5µls of each sporangia dilution was used as template for PCR amplification using primers PLAS17F and PLAS15R (see details in Example 1).

A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3. The PCR conditions and components were as described in previous Examples. Upon gel electrophoresis analysis of the PCR products, an expected size band (~500bp) was found. This PCR product was then cloned in the Invitrogen pCR2.1 TA vector and five resulting clones with the correct size inserts (~500bp) were sequenced using the M13 forward and reverse primers. Upon analysis of the sequence data using the relevant bioinformatics software, it was found that the resulting sequence encoded a novel cytochrome *b* gene sequence with close homology to other oomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S. cerevisiae* amino acid numbering system) can be found in table 3.

EXAMPLE 9:

In example 9 we report the characterisation of partial *Mycosphaerella graminicola* cytochrome *b* gene sequence. *M. graminicola* is the causal agent of leaf blotch on wheat.

Infected wheat leaves were collected from the field and incubated in a humid environment to promote spore production. Cirri were removed from leaves and spread onto Czapek Dox V8 agar plates and incubated in a controlled environment at 19°C for 6 days. Single colony isolates were further subcultured and bulked up by shake flask culture in a suitable medium. Material was removed and maintained at -80°C until needed. A genomic DNA preparation was carried out as described in Example 1 on 200mg of ground mycelia. The genomic DNA yield was analysed by gel electrophoresis and diluted 1:10 and 1:100 in ddH_20. 5µls of each dilution was used as template for PCR amplification using primers Cyt3F and Cyt9R (details in Example 2). PCR components and conditions were are described in Example 2. The PCR products were analysed by gel electrophoresis and a band of the correct size was found (~500bp). This PCR product was cloned in TA Invitrogen pCR2.1 vector and 5 clones containing correct size inserts were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found to encode a novel cytochrome b gene sequence which showed close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

EXAMPLE 10:

In example 10 we report the characterisation of partial *Colletotrichum graminicola* cytochrome *b* gene sequence. *C. graminicola* is the causal agent of cereals and grasses anthracnose.

Infected leaf material (turf or moss) was collected from the field and the fungal

out as described in Example 1 on 200mg of ground mycelia. The genomic DNA yield

was analysed by gel electrophoresis

dilution was used as template for PCR amplification using primers deg4F and deg3R (details in Example 3). PCR components and conditions were are described in Example 3. The PCR products were analysed by gel electrophoresis and a band of the correct size was found (~500bp). This PCR product was cloned in TA Invitrogen pCR2.1 vector and 5 clones containing correct size inserts were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found to encode a novel cytochrome *b* gene sequence which showed close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.
degenerate primer pair (see details in Example 3).

EXAMPLE 11:

In example 11 we report the characterisation of partial *Colletotrichum gloeosporioides* cytochrome *b* gene sequence. *C. gloeosporioides* is the causal agent of fruit anthracnose (e.g. pepper, avocado and mango)
Infected plant material (mango or chilli) was collected from the field and the fungal material removed and subcultured on artifical media. Mycelia was bulked up in shake flask culture and harvested by filtration through Miracloth. The mycelia were stored at -80°C until needed. The mycelia were ground using a sterile pestle and mortar and 100mg was used in a genomic DNA preparation and a first strand cDNA synthesis (procedures as described in previous examples). 5uls of each genomic dilutions and neat cDNA was used as template for PCR amplification using primers deg4F and deg3R (details in Example 3). PCR components and conditions were are described in Example 3. The PCR products were analysed by gel electrophoresis and bands of the correct size were found (~500bp). The PCR product was the same size when using genomic DNA or cDNA as template which indicates the lack of introns in the DNA region amplified. The PCR products were cloned in TA Invitrogen pCR2.1 vector and 5 clones containing correct size inserts for each cloning event were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found to be identical in both cases and to

encode a novel cytochrome *b* gene sequence which showed close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

EXAMPLE 12:

In example 12 we report the characterisation of partial *Oidium lycopersicum* cytochrome *b* gene sequence. *O. lycopersicum* is the causal agent for tomato powdery mildew.

Diseased tomato leaves were collected from the field and conidia subcultured to fresh leaf material by dry inoculation in a settling tower. Conidia growing from the resulting infection were removed by aspiration by vacuum pump into a sterile Eppendorf tube and kept in at -80C until needed. A first strand cDNA synthesis was carried out on 100mg of spores as described previously and 5uls was used for PCR amplification using primers Ery2-4 (details in example 6). The PCR components and conditions were as described previously. When the PCR products were analysed by gel electrophoresis, a PCR product was found at the expected size (~500bp). This PCR product was cloned and 5 clones containing the correct size insert (~500bp) were sequenced as described previously. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found encode a novel cytochrome *b* gene sequence which showed close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

EXAMPLE 13:

In example 13 we report the characterisation of partial *Leveillula taurica* cytochrome *b* gene sequence. *O. lycopersicum* is the causal agent for tomato powdery

inoculated to fresh leaf material on whole plants. Conidia from resulting infections

for PCR analysis. Diseased pepper and tomato leaves were collected from the field and glasshouse at Jealott's Hill and infected leaf material submitted directly for genomic DNA extraction and PCR analysis..

The cDNA sequence encoding for 30bp upstream and downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table x.

EXAMPLE 14:

In example 14 we report the characterisation of partial *Alternaria solani* cytochrome *b* gene sequence. *A. solani* is the causal agent for early blight in tomato and potato.

Infected leaf material (tomato or potato) was collected from the field and the fungal material removed and subcultured on artifical media. Material was bulked up in shake flask culture. Culture collection isolates had been stored in liquid nitrogen and periodically subcultured on artificial media or passaged through live host material before re-storing at -80C. Mycelia grown in a shake flask were ground using a sterile pestle and mortar and 100mg was used in a genomic DNA preparation and a first strand cDNA synthesis (procedures as described in previous examples). 5uls of each genomic dilutions and neat cDNA was used as template for PCR amplification using primers deg4F and deg3R (details in Example 3). PCR components and conditions were are described in Example 3. The PCR products were analysed by gel electrophoresis and bands of the correct size were found (~500bp). The PCR product was the same size when using genomic DNA or cDNA as template which indicates the lack of introns in the DNA region amplified. The PCR products were cloned in TA Invitrogen pCR2.1 vector and 5 clones containing correct size inserts for each cloning event were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found to be identical in both cases and to encode a novel cytochrome *b* gene sequence which showed close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

A sequence only 12bp different from the previously isolated *A. solani* sequence was also amplified when using genomic DNA extracted directly from field infected tomato leaves as template for PCR amplification using primers Ery11-12 (see above for details). Although this DNA sequence had 12 bp difference to the previously isolated *A. solani* sequence this did not result in any difference at the amino acid level when the two sequences were translated. The significant difference in the two sequences was the presence of an ~1.2kb intron 61bp downstream of the second base in the G_{143} codon.

EXAMPLE 15:

In example 15 we report the characterisation of partial *Cercospora arachidola* cytochrome *b* gene sequence. *C. arachidola* is the causal agent of peanut leaf blotch.

Infected leaf material (peanut) was collected from the field and the fungal material removed and subcultured on artifical media. Cultures were stored in liquid nitrogen until required. Material was removed from storage onto agar and resulting colonies bulked up in shake flask culture and kept at -80C until needed. Mycelia were ground using a sterile pestle and mortar and 100mg was used in a genomic DNA preparation and a first strand cDNA synthesis (procedures as described in previous examples). 5uls of each genomic dilutions and neat cDNA was used as template for PCR amplification using primers deg4F and deg3R (details in Example 3). PCR components and conditions were are described in Example 3. The PCR products were analysed by gel electrophoresis and bands of the correct size were found (~500bp). The PCR product was the same size when using genomic DNA or cDNA as template which indicates the lack of introns in the DNA region amplified. The PCR products were cloned in TA Invitrogen pCR2.1 vector and 5 clones containing correct size inserts for each cloning event were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found to be identical in both cases and to encode a novel cytochrome *b*

second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3

EXAMPLE 16:

In example 16 we report the characterisation of partial *Rhizoctonia solani* cytochrome *b* gene sequence. *R. solani* is the causal agent of root stem rot or damping off.

Infected leaf material (rice) was collected from the field and the fungal material removed and subcultured on artifical media. Cultures were stored in liquid nitrogen until required. Material was removed from storage onto agar and resulting colonies bulked up in shake flask culture and kept at -80C until needed. A first strand cDNA synthesis was carried out on 100mg of ground mycelia as described previously and 5uls was used for PCR amplification using basidiomycete degenerate primers 1F (5' WYTRGTAYTAATGATGGCTATHGG 3') and 1R (5' TCTTARWATWGCATAGAAWGG 3') which delineate amino acids 121 to 283 according to the *S. cerevisae* numbering system. The PCR components and conditions were as described previously. When the PCR products were analysed by gel electrophoresis, a PCR product was found at the expected size (~500bp). This PCR product was cloned and 5 clones containing the correct size insert (~500bp) were sequenced as described previously. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found encode a novel cytochrome *b* gene sequence which showed close homology to other basidiomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

EXAMPLE 17:

In example 17 we report the characterisation of partial *Pythium aphanidermatum* cytochrome *b* gene sequence. *P. aphanidermatum* is the causal agent of damping off.

Infected leaf material (turf) was collected from the field and the fungal material removed and subcultured on artifical media. Cultures were stored in liquid nitrogen until required. Material was removed from storage onto agar and resulting colonies bulked up in shake flask culture and kept at -80C until needed. A genomic

DNA preparation was carried out as described in Example 1 on 200mg of ground mycelia. The genomic DNA yield was analysed by gel electrophoresis and diluted 1:10 and 1:100 in dd$H_2$0. 5uls of each dilution was used as template for PCR amplification using primers PLAS17F and PLAS15R (details in Example 1). PCR components and conditions were are described in Example 1. The PCR products were analysed by gel electrophoresis and a band of the correct size was found (~500bp). This PCR product was cloned in TA Invitrogen pCR2.1 vector and 5 clones containing correct size inserts were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the sequence was found to encode a novel cytochrome *b* gene sequence which showed close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in table 3.

EXAMPLE 18

In example 18 we report the characterisation of partial *Mycosphaerella musicola* cytochrome b geen sequence. *M. musicola* is the causal agent of Yellow Sigatoka disease on banana.

Infected banana leaves were collected from the field and ascospores inoculated from leaves directly onto artificial media in a petri dish. Monoascosporic isolates were maintained on artificial media and prepared for PCR analysis by shake flask culture in a broth medium. Mycelia were collected through Miracloth and ground to a fine powder using a sterile pestle and mortar. 100mg of ground mycelia were used in a genomic DNA extraction and in a first strand cDNA synthesis (as described in Example 7). The genomic DNA was diluted 1:10, 1:100 and 1:1000 prior to use as PCR template. 5µls of cDNA and 10µls of each genomic dilution were used as template for PCR amplification using the degenerate primer pair F4/R3 as described in Example 3. The PCR conditions and components were as described in [illegible]

[illegible] template. The two PCR products from both templates, were cloned in the Invitrogen [illegible]

were sequenced using the M13 forward and reverse primers. Upon analysis of the sequence data using the relevant bioinformatics software, it was found that the resulting sequence encoded a novel cytochrome *b* gene sequence with close homology to other Ascomycete cytochrome *b* gene sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the amino acid numbering system) can be found in table 3.

CLAIMS

1. A method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

2. A method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to the presence or absence of said mutation in said nucleic acid.

3. A method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with an appropriate diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended either when the said mutation is present in the sample or when wild type sequence is present; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

nucleic acid with a diagnostic primer for the specific mutation in the presence

that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

5. A method according to any of the preceding claims wherein the mutation is present in a fungal cytochrome *b* gene where said mutation results in the inhibition of a strobilurin analogue or any other compound in the same cross resistance group to the active site of the cytochrome b protein but still allows the respiration process to occur.

6. A method according to the previous claim wherein the mutation in the fungal nucleic acid results in the replacement of a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the encoded protein with an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid and alanine.

7. A method according to any of the preceding claims wherein the mutation in the fungal nucleic acid results in the replacement of a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the encoded protein with an alanine.

8. A method according to claim 2 for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein wherein said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of said mutation in said nucleic acid.

9. A method according to claim 3 for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the mutation resulting in a $G_{143}A$ replacement in the encoded protein in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when a mutation is present in the sample resulting in a $G_{143}A$ replacement in the encoded protein; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

10. A method according to any of the preceding claims wherein the fungal gene is present in a plant pathogenic fungus selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporiodes, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Psuedoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

11. A method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide

acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein

12. A method according to claim 11 wherein the said single nucleotide polymorphism occurs at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

13. A method according to any of the preceding claims wherein the single nucleotide polymorphism which is detected is a G to C base change occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

14. A fungal DNA sequence encoding all or part of a cytochrome *b* protein wherein said DNA sequence encodes a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 and is obtainable from a fungus slected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, , Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

15. A fungal DNA sequence according to claim 14 comprising all or part of a DNA sequence selected from the group ;

5'TTTTGCCTTGGGGACAAATGAGTTTTTGGGGTGCAACAGTTATTACAAATTTATT CTCGGC 3'

5'TATTGCCATACGGGCAGATGAGCCACTGGGGTGCAACCGTTATCACTAACCTAA TGAGCGC 3'-

5'TGCTTCCTTATGGACAGATGTCTTTATGAGGTGCCACAGTTATAACTAATCTTAT GAGTGC 3'-

5'TTTTACCCTACGGGCAAATGAGCCTTTGAGGTGCTACAGTTATTACTAACCTTAT
GAGTGC 3'-
5'TTTTACCCTACGGGCAAATGAGCCTTTGAGGTGAAATATTTGCCTCAAATGTAT
AACTAAT 3'-
5'TATTACCTTATGGTCAAATGTCTTTATGAGGAGCAACAGTTATAACTAACTTATT
GAGTGC 3'
5'TTTTACCTTATGGTCAAATGTCTTTATGAGGAGCTACAGTTATAACTAATTTAAT
GAGCGC 3'-
5'TACTTCCCTTCGGTCAAATGTCGCTCTGGGGTGCAACCGTTATTACTAACCTTAT
GAGCGC 3'-
5' *TCTGGGGTGCAACCGTTAAGTAATAGCGGTTGTAAAA-
5'TTTTACCCTACGGGCAGATGAGCCTATGGGGTGCAACCGTTATTACTAACCTTAT
GAGCGC 3'-
5'*AGCCTATGGGGTGCAACCGTTAAGTAGGTAATAGCGGTTGA 3'-
5'TTTTACCTTACGGACAAATGTCATTATGAGGTGCTACAGTTATTACTAACCTTAT
AAGTGC 3'-
5'TATTACCTTGGGGTCAAATGAGTTTTTGGGGTGCTACTGTTATTACTAATTTATT
TTCAGC 3'-
5'TTTTACCTTATGGACAAATGTCATTATGAGGTGCAACAGTTATTACTAACCTTAT
AAGTGC 3'-
5'TTTTACCCTACGGGCAGATGAGCCTGTGGGGTGCAACCGTTATTACTAACCTTAT
GAGCGC 3'-
5'TTTTACCATACGGACAAATGTCATTATGAGGTGCAACAGTTATTACTAACCTTAT
GAGTGC 3'-
5'TTTTACCTTGGGGACAAATGAGTTTTTGGGGTGCAACTGTTATTACTAATTTATT
TTCTGC 3'-
5'TTCTTCCTTATGGGCAAATGTCTTTATGAGGTGCTACAGTTATTACTAACCTTAT
GAGTGC 3'-
5'TATTACCTTATGGACAAATGTCATTATGAGGAGCTACAGTTATTACTAATTTATT
ATCTGC 3'-
5'TGCTTCCATACGGGCAAATGTCTCTGTGGGGTGCTACAGTAATTACTAATTTACT
TTCTGC 3'-
5'TTTTACCTTATGGTCAAATGTCTTTATGAGGAGCTACAGTTATAACTAATTTAAT

A fungal DNA sequence encoding all or part of a cytochrome P protein which, when said sequence is lined up against the corresponding wild type DNA

nucleotide polymorphism mutation at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

17. A fungal DNA sequence according to claim 16 wherein said single nucleotide polymorphism mutation occurs at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

18. A fungal DNA sequence encoding all or part of a cytochrome *b* protein wherein said DNA sequence encodes an alanine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 and is obtainable from a fungus slected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

19 A fungal DNA sequence encoding all or part of a cytochrome *b* protein according to claim 18 wherein said DNA sequence contains a single nucleotide polymorphism which results in the replacement of the normal guanine reside with a cytosine residue at a position in the DNA corresponding

to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

20. A fungal DNA sequence according to claims 16 to 19 comprising all or part of a sequence selected from the group

5'TTTTGCCTTGGGGACAAATGAGTTTTTGGGCTGCAACAGTTATTACAAATTTATT CTCGGC 3'

5'TATTGCCATACGGGCAGATGAGCCACTGGGCTGCAACCGTTATCACTAACCTAA TGAGCGC 3'-

5'TGCTTCCTTATGGACAGATGTCTTTATGAGCTGCCACAGTTATAACTAATCTTAT GAGTGC 3'-

5'TTTTACCCTACGGGCAAATGAGCCTTTGAGCTGCTACAGTTATTACTAACCTTAT GAGTGC 3'-

5'TTTTACCCTACGGGCAAATGAGCCTTTGAGCTGAAATATTTGCCTCAAATGTATA ACTAAT 3'-

5'TATTACCTTATGGTCAAATGTCTTTATGAGCAGCAACAGTTATAACTAACTTATT GAGTGC 3'

5'TTTTACCTTATGGTCAAATGTCTTTATGAGCAGCTACAGTTATAACTAATTTAAT GAGCGC 3'-

5'TACTTCCCTTCGGTCAAATGTCGCTCTGGGCTGCAACCGTTATTACTAACCTTAT GAGCGC 3'-

5' *TCTGGGCTGCAACCGTTAAGTAATAGCGGTTGTAAAA-

5'TTTTACCCTACGGGCAGATGAGCCTATGGGCTGCAACCGTTATTACTAACCTTAT GAGCGC 3'-

5'*AGCCTATGGGCTGCAACCGTTAAGTAGGTAATAGCGGTTGA 3'-

5'TTTTACCTTACGGACAAATGTCATTATGAGCTGCTACAGTTATTACTAACCTTAT AAGTGC 3'-

5'TATTACCTTGGGGTCAAATGAGTTTTTGGGCTGCTACTGTTATTACTAATTTATT TTCAGC 3'-

5'TTTTACCTTATGGACAAATGTCATTATGAGCTGCAACAGTTATTACTAACCTTAT AAGTGC 3'-

[illegible] GAGTGC 3'-

5'TTTTACCTTGGGGACAAATGAGTTTTTGGGCTGCAACTGTTATTACTAATTTATT TTCTGC 3'-
5'TTCTTCCTTATGGGCAAATGTCTTTATGAGCTGCTACAGTTATTACTAACCTTAT GAGTGC 3'-
5'TATTACCTTATGGACAAATGTCATTATGAGCAGCTACAGTTATTACTAATTTATT ATCTGC 3'-
5'TGCTTCCATACGGGCAAATGTCTCTGTGGGCTGCTACAGTAATTACTAATTTACT TTCTGC 3'-
5'TTTTACCTTATGGTCAAATGTCTTTATGAGCAGCTACAGTTATAACTAATTTAAT GAGTGC 3'

21. A fungal cytochrome *b* protein which confers fungal resistance to a strobilurin analogue or a compound within the same cross resistance group wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that protein is not the *Mycena galopoda* cytochrome *b* protein.

22. A fungal cytochrome *b* protein according to claim 21 wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that protein is not the *Mycena galopoda* cytochrome *b* protein.

23. A method for the detection of a mutation in fungal cytochrome *b* gene resulting in the replacement of a glycine residue in the encoded protein at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 said method comprising identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from

around 30 to 90 nucleotides upstream and/or downstream of the position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

24. A method according to claim 23 wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

25. An allele specific oligonucleotide capable of binding to a fungal DNA sequence encoding a wild type cytochrome *b* protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said oligonucleotide comprises a sequence which recognises a DNA sequence encoding a glycine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 .

26. An allele specific oligonucleotide capable of binding to a fungal DNA sequence encoding a mutant cytochrome *b* protein wherein said oligonucleotide comprises a sequence which recognises a DNA sequence

corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 .

27. A diagnostic primer or a diagnostic oligonucleotide capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer or oligonucleotide corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and the presence of said nucleotide gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group.

28. A diagnostic primer according to claim 27 wherein either the penultimate nucleotides (-2) or (-3) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

29. One or more diagnostic primers for detecting a $G_{143}A$ mutation in a fungal cytochrome *b* gene selected from the group consisting of

5'CCTTGGTGACAAATGAGTTTTTGG**A**C3'
5'CCATACGGGCAGATGAGCCACTGG**A**C3'
5'CCTTATGGACAGATGTCTTTATGA**T**C3'
5'CCCTACGGGCAAATGAGCCTTTG**C**GC3'
5'CCTTATGGTCAAATGTCTTTATGA**A**C3'
5'CCTTATGGTCAAATGTCTTTATGA**T**C3'
5'CCCTTCGGTCAAATGTCGCTCTGG**A**C3'
5'CCCTACGGGCAGATGAGCCTATGG**T**C3'
5'CCTTACGGACAAATGTCATTATGA**A**C3'
5'CCTTGGTGTCAAATGAGTTTTTGG**A**C3'
5'CCTTATGGACAAATGTCATTATGA**A**C3'
5'CCCTACGGGCAGATGAGCCTGTGG**A**C3'
5'CCATACGGACAAATGTCATTATGA**A**C3'
5'CCTTGGGGACAAATGAGTTTTTGG**A**C3'
5'CCTTATGGGCAAATGTCTTTATGA**A**C3'
5'CCTTATGGACAAATGTCATTATGA**A**C3'
5'CCATACGGGCAAATGTCTCTGTGG**A**C3'
5'GTGTATGGTCAAATGAGCCTATGG**C**C3'
5'CCTTATGGACAGATGTCATTATGA**A**C3'
5'CCTTGGGGACAAATGAGTTTTTGG**A**C3'
5'CCTTATGGTCAAATGTCTTTATGA**T**C3'

and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given above and wherein up to 10, such as up to 8, 6, 4, 2, 1, of

the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer.

30. An allele specific oligonucleotide probe capable of detecting a fungal cytochrome *b* gene polymorphism at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome b residue 143 in the protein as defined by the positions in EMBL ACCESSION NO. X84042.

31. An allele specific oligonucleotide probe according to claim 30 wherein said polymorphism is at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome b residue 143 in the protein as defined by the positions in EMBL ACCESSION NO. X84042.

32. An allele specific oligonucleotide probe according to claim 31 wherein the polymorphism is a guanine to cytosine base change.

33. A diagnostic kit comprising one or more of the diagnostic primers as claimed in claims 27 to 29, or an allele specific oligonucleotide as claimed in claims 25 or 26 or an allele specific oligonucleotide probe as claimed in claim 30 to 32, nucleotide triphosphates, polymerase, and buffer solution.

34. A method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting a mutation in a fungal gene wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is

35. A method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene.

36. A method of detecting plant pathogenic fungal resistance to a fungicide according to claim 34 or claim 35 said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation the presence of which gives rise to fungicide resistance in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

37. A method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene said method comprising detecting the presence or absence of a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying and quantifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

38. A method according to claim 37 said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid

with appropriate primers in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to both the presence and absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene, and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of an amplicon generated during the PCR reaction.

39. A method according to claim 37 or claim 38 comprising contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to fungicide resistance to a fungicide whose target protein is encoded by a mitochondrial gene, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

40. A method of selecting an active fungicide and optimal application levels thereof for application to a crop comprising analysing a sample of a fungus capable of infecting said crop and detecting and/or quantifying the presence and/or absence of a mutation in a gene from said fungus wherein the presence of said mutation may give rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene and then selecting an active fungicide and optimal application levels thereof

single nucleotide polymorphism detection technique.

42. A method according to claim 40 or claim 41 wherein the detection method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

43. A method of controlling fungal infection of a crop comprising applying a fungicide to the crop wherein said fungicide is selected according to any of claims 40 to 42.

44. A method according to claims 34 to 43 wherein the fungicide is a strobilurin analogue or any other compound in the same cross resistance group.

45. A method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome b protein.

46. A method for detecting fungal resistance to a strobilurin analogue according to claim 45 said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome b protein.

47. A method according to any of the preceding claims wherein the method of detection and/or quantifying is based on fluorescence detection of diagnostic primer extension products.

48. A method according to any of the preceding claims wherein the method of detection involves the use of the Scorpion™ detection system.

49. A method according to any of claims 34 to 48 wherein said mutation occurs at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 in the cytochrome b protein.

50. A method according to any of claims 34 to 49 wherein the said mutation is a guanine to cytosine change resulting in a G143A replacement in the encoded protein where a wild type glycine residue is substituted with an alanine residue at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143.

51. A computer readable medium having stored thereon any of the sequences in any of the previous claims including all or part of a DNA sequence or protein sequence encoding a mutant cytochrome *b* protein as herein described wherein the presence of a mutation gives rise to fungal resistance to a strobilurin analogue or any compound in the same cross resistance group; all or part of a DNA or protein sequence encoding a wild type cytochrome *b* sequence from a fungus selected from the group *Plasmopara viticola, Erysiphe graminis f.sp tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella*

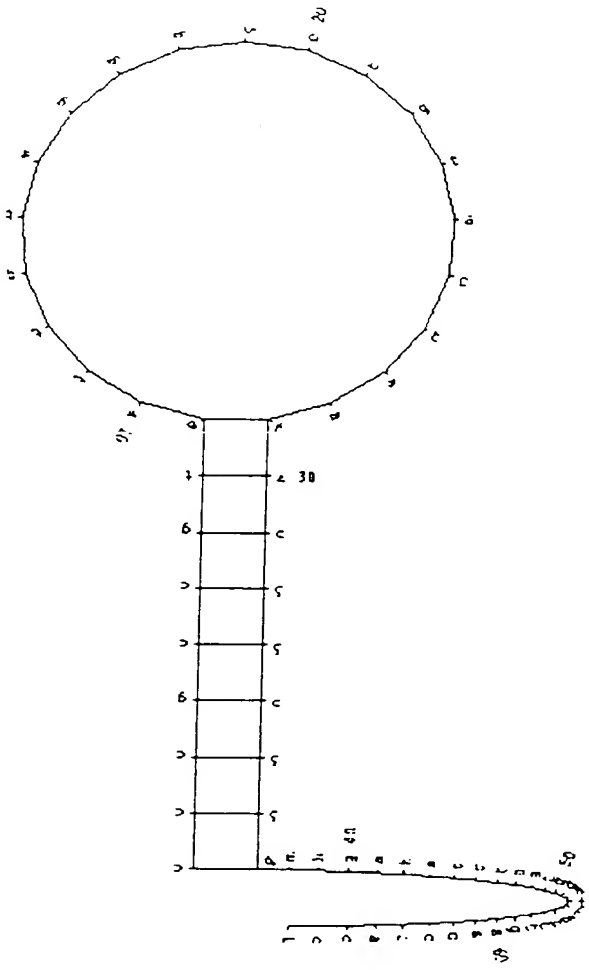
Pythium aphanidermatum, Colletotrichum glocosporioides, Oidium

taurica, Pseudoperonospora cubensis, Alternaria solani. Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola; or any allele specific oligonucleotide; allele specific primer, allele specific oligonucleotide probe, common or diagnostic primer according to any of the preceding claims..

52. A diagnostic kit for use in a method according to any of claims 1 to 13, 21 or 34 to 50.

53. A diagnostic kit according to claim 52 comprising one or more of the following: diagnostic, wild type, control and/or common oligonucleotide primers, allele specific oligonucleotide primers, allele specific oligonucleotide probes, appropriate nucleotide triphosphates, for example dATP, dCTP, dGTP, dTTP, a suitable polymerase, and a buffer solution.

Figure 1 *P. viticola* Scorpion primer folding



The *P. viticola* Scorpion primer folds in this configuration with an energy of -2.2 in its inert form.

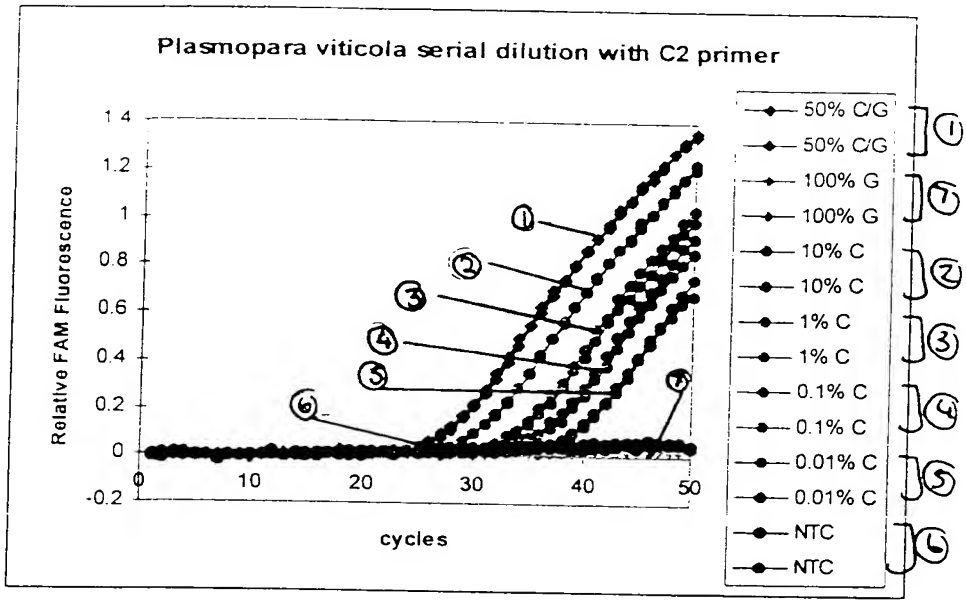


Figure 2a

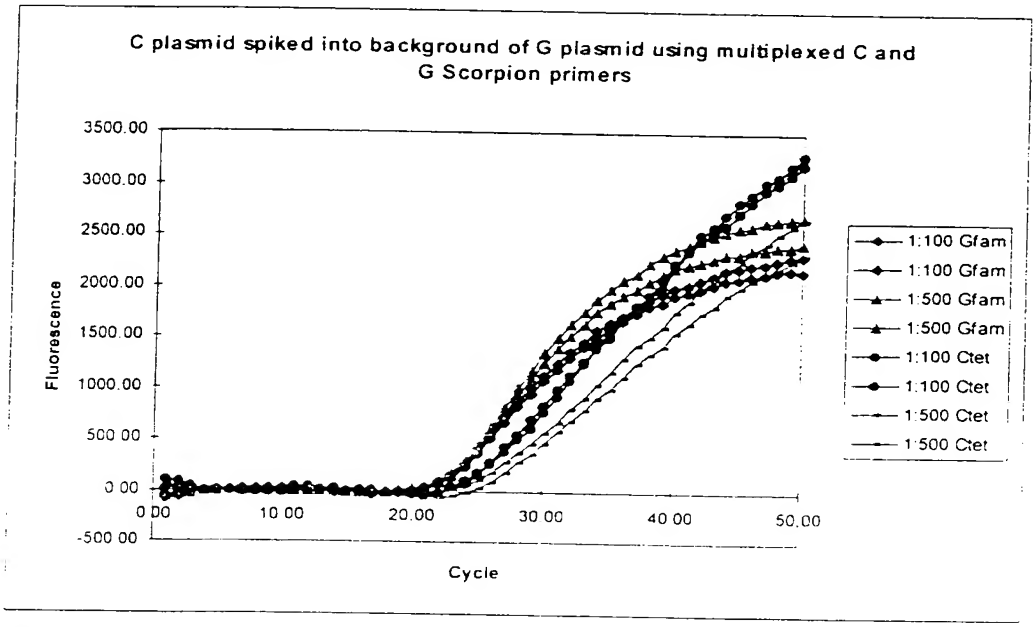


Figure 2b

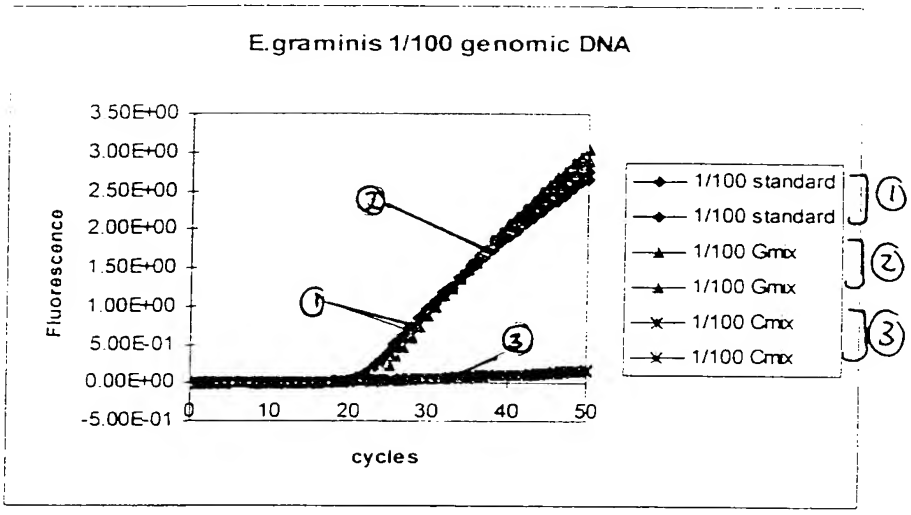


Figure 3a

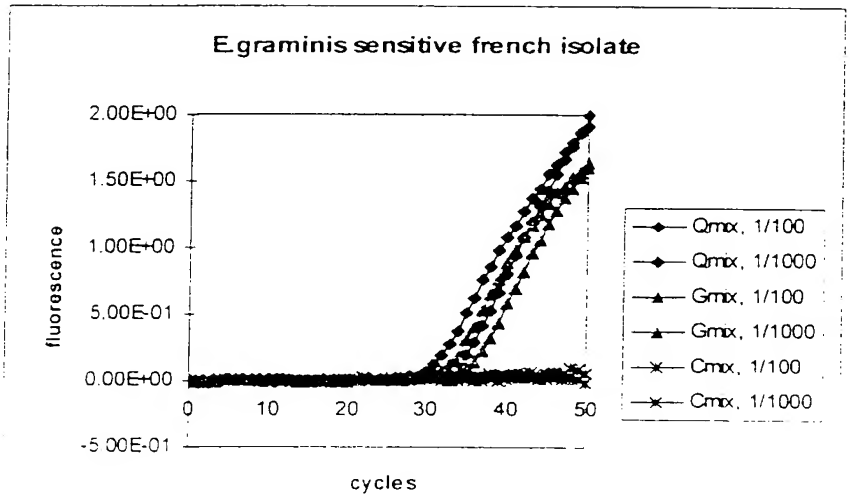
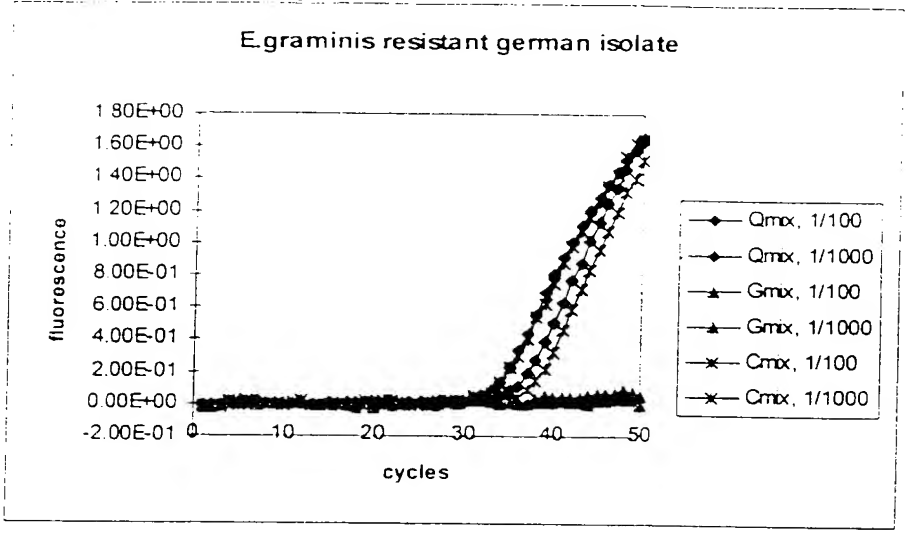


Figure 3b


E.graminis resistant german isolate
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1.60E+00
1.40E+00
1.20E+00
1.00E+00
8.00E-01
6.00E-01
4.00E-01
2.00E-01
0.00E+00
-2.00E-01
fluorosconco
0
10
20
30
40
50
cycles
Qmax, 1/100
Qmax, 1/1000
Gmax, 1/100
Gmax, 1/1000
Cmax, 1/100
Cmax, 1/1000

Figure 4

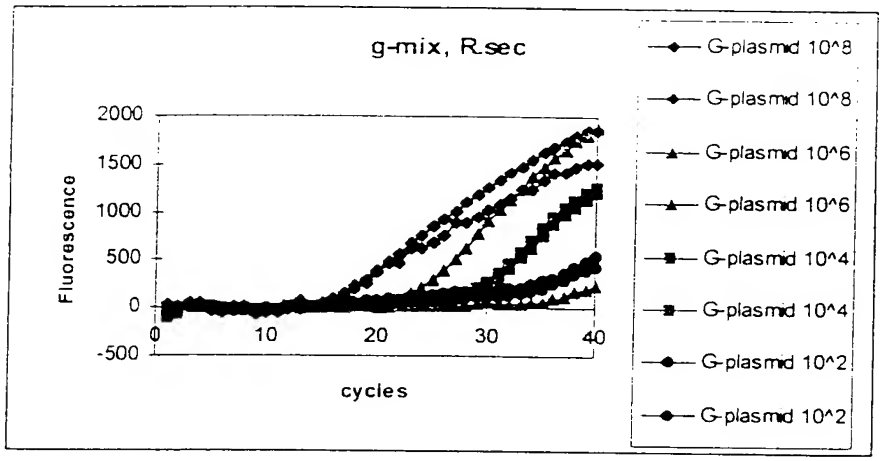


Figure 5a

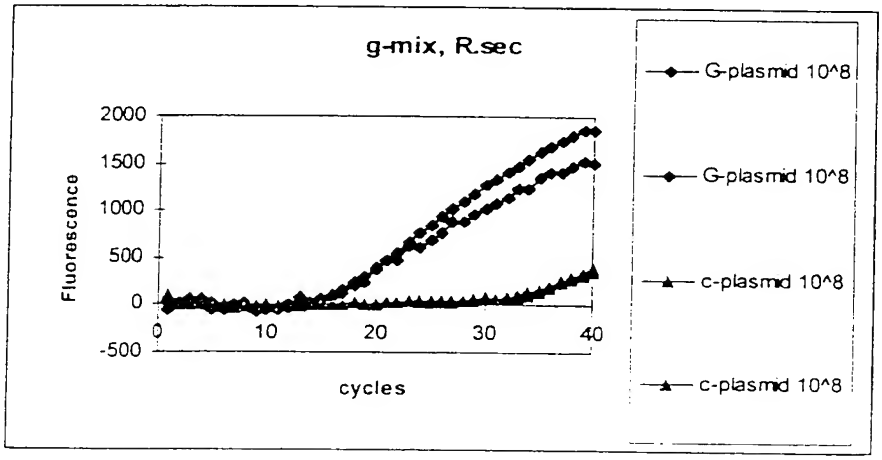


Figure 5b

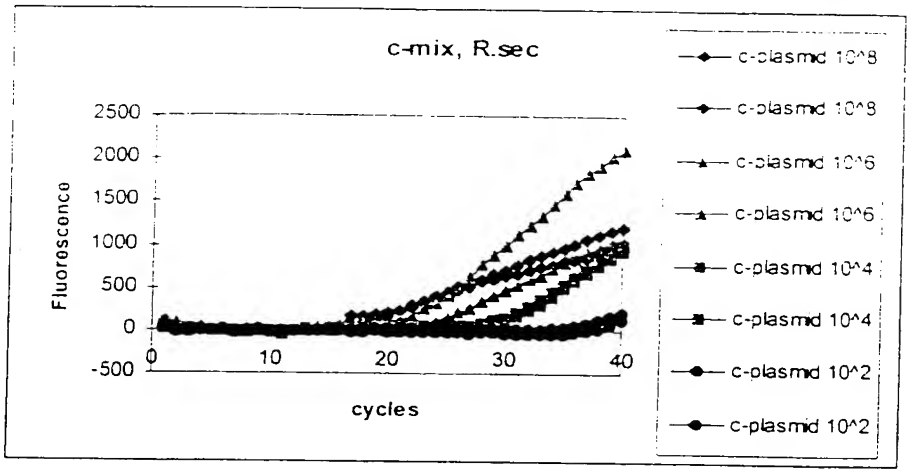


Figure 6a

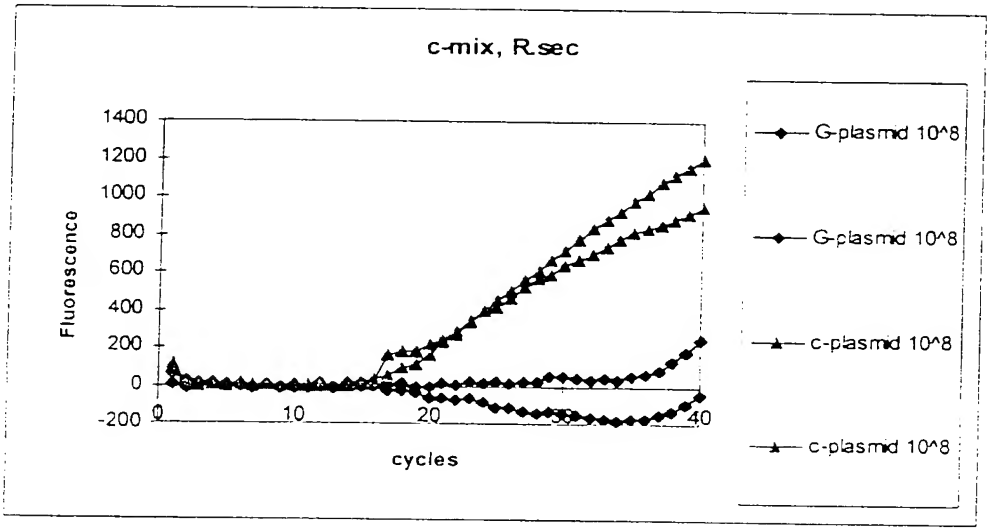
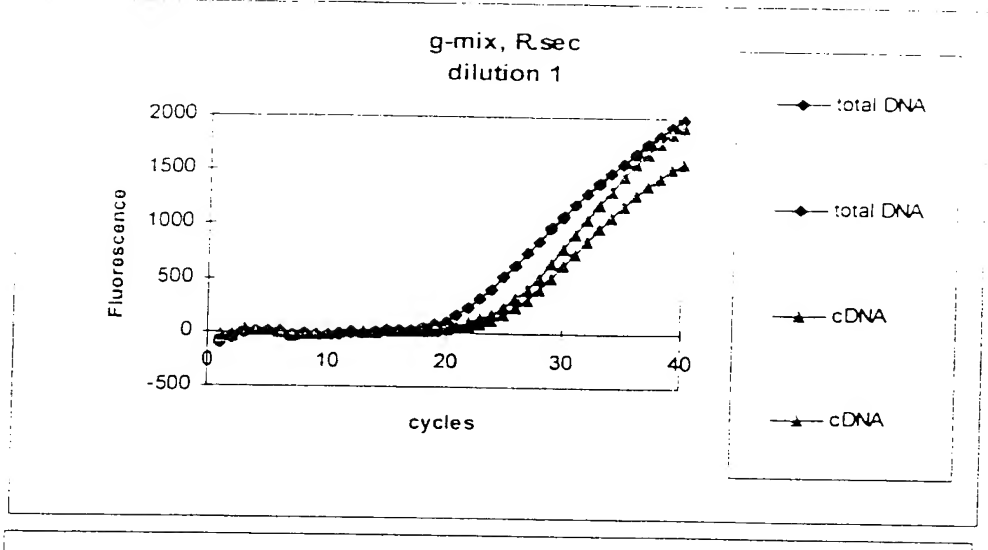
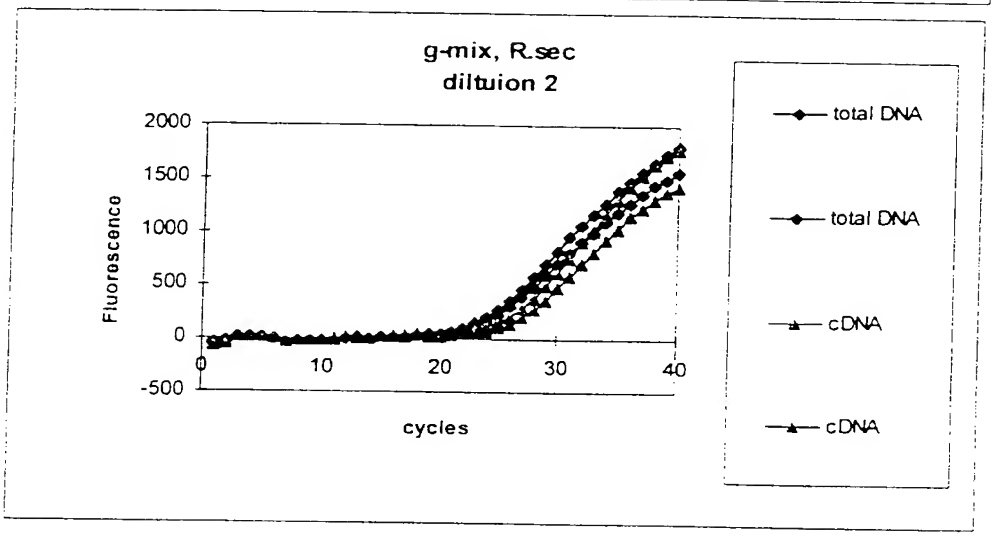


Figure 6b





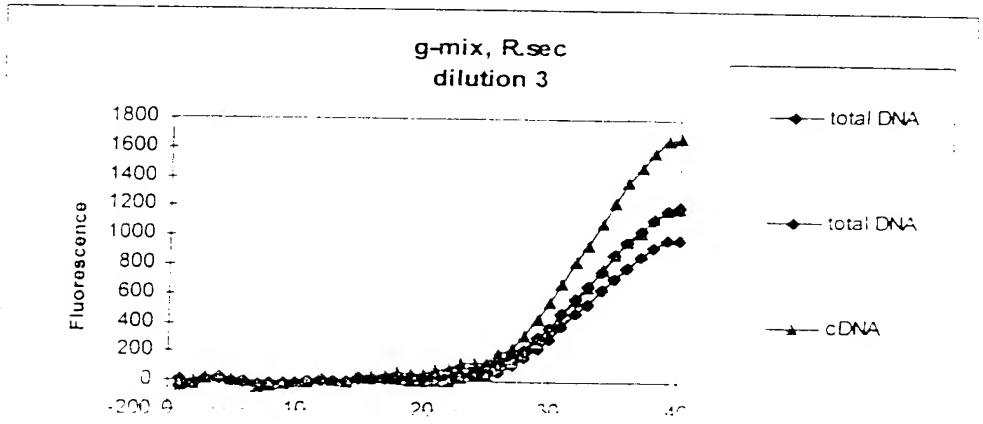
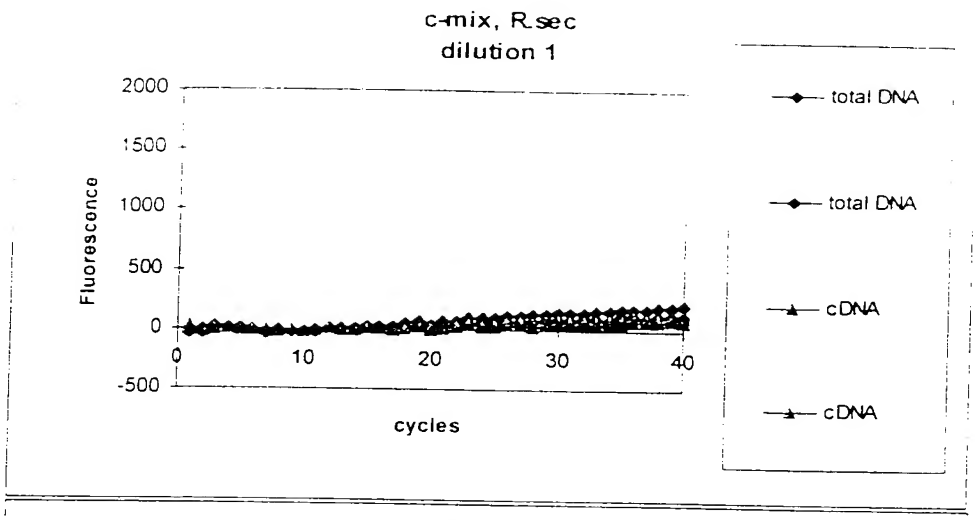


Figure 7a, b and c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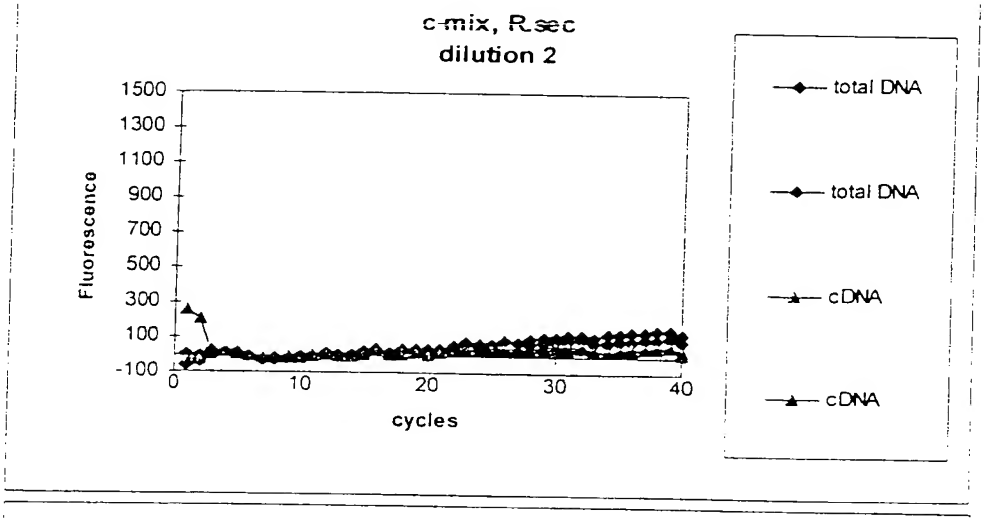
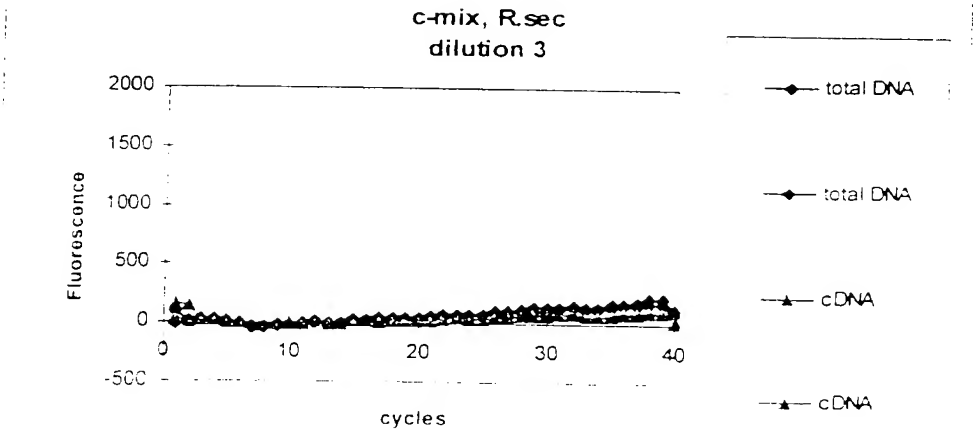
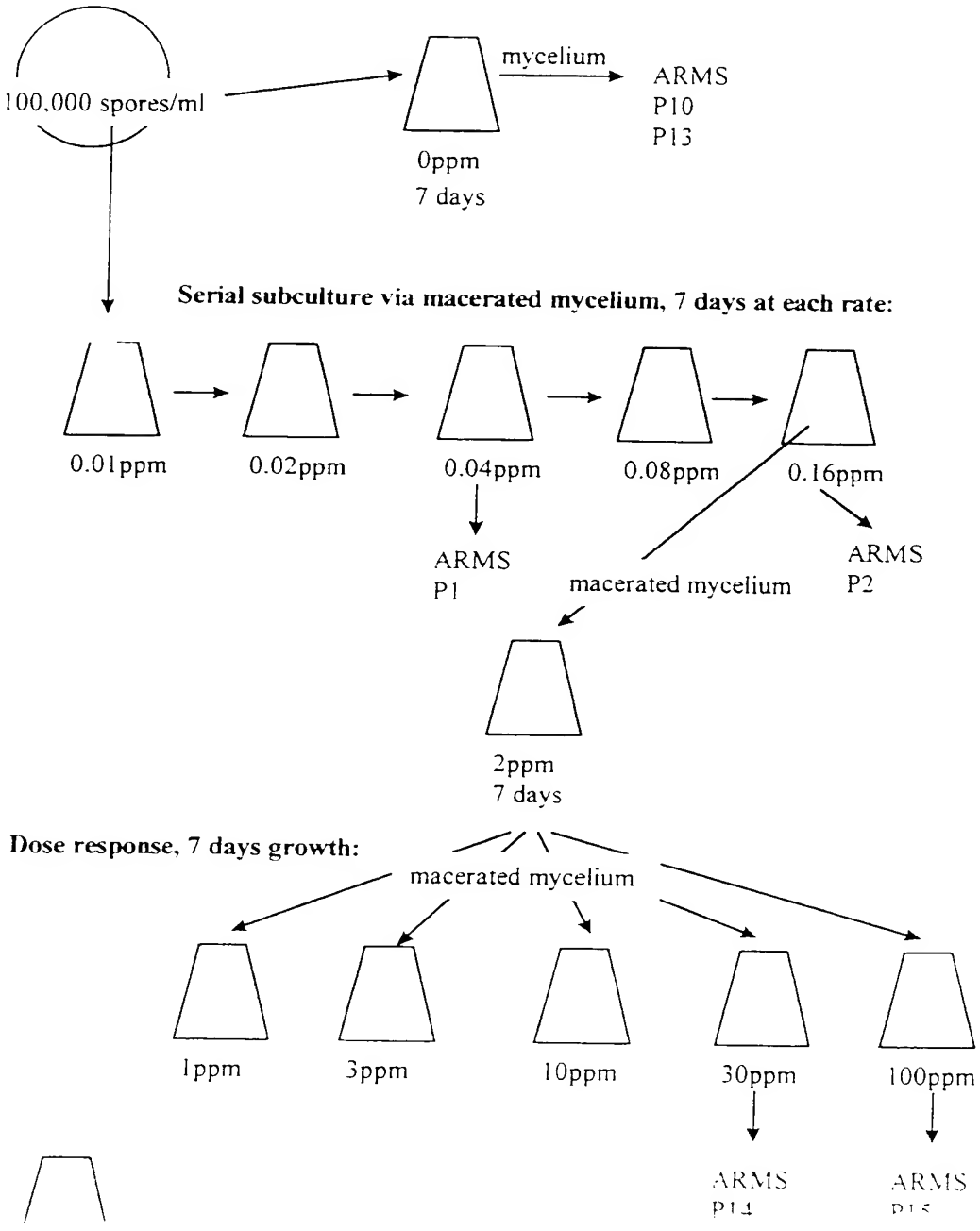
1500
1000
500
0
-500
Fluorescence
0
10
20
30
40
cycles
total DNA
total DNA
cDNA
cDNA


c-mix, R.sec
dilution 2
1500
1300
1100
900
700
500
300
100
-100
Fluorescence
0
10
20
30
40
cycles
total DNA
total DNA
cDNA
cDNA


c-mix, R.sec
dilution 3
2000
1500
1000
500
0
-500
Fluorescence
0
10
20
30
40
cycles
total DNA
total DNA
cDNA
cDNA

Preparation of *Pyrenophora teres* isolate K1916 for ARMS assay
(Selection in strobilurin analogue 2-amended alkyl



Preparation of *Pyrenophora teres* isolates for ARMS assay
(Selection in strobilurin analogue 2-amended alkyl

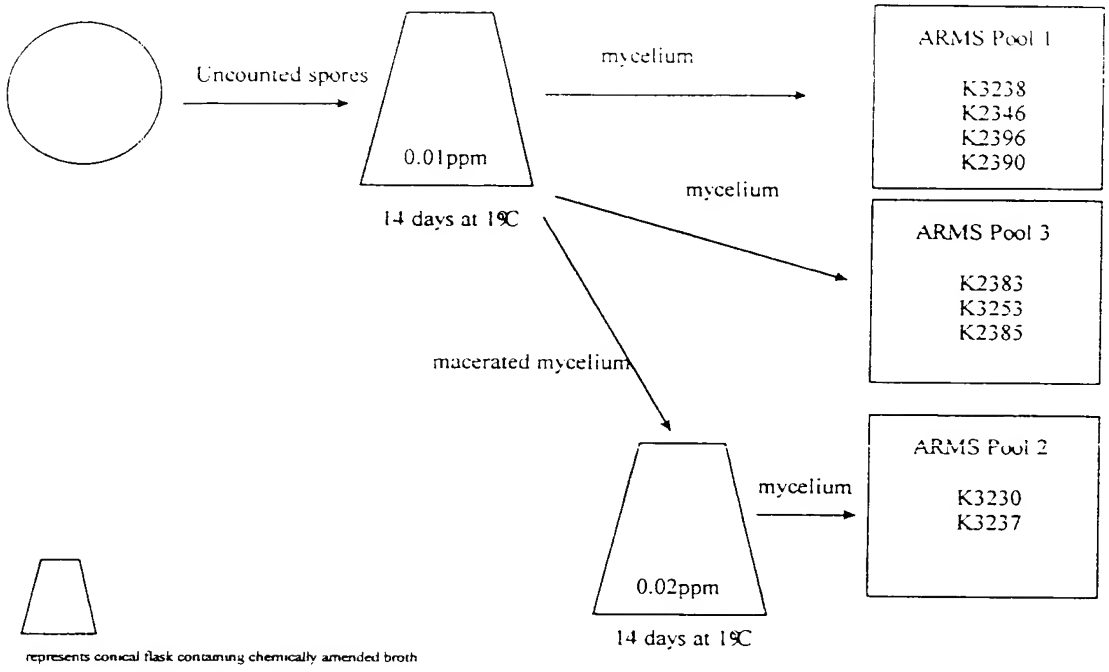
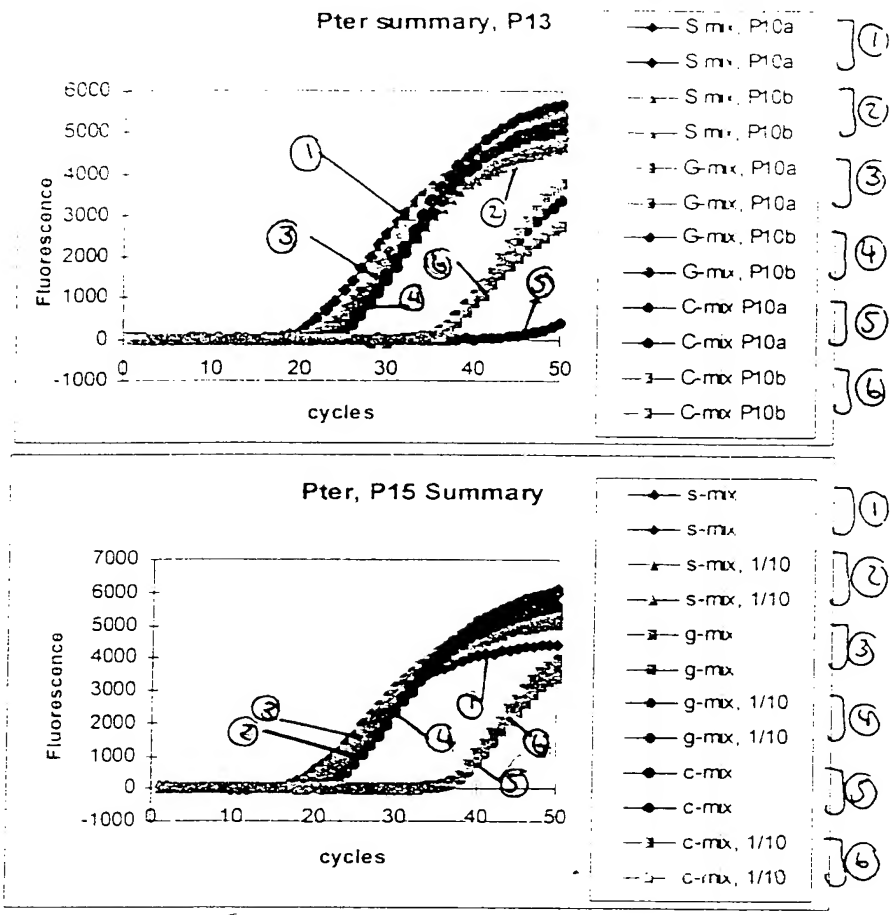


Figure 9b



Figures 10 a and b